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Regulation of Neurite Outgrowths by the Pseudophosphatase MK-STYX in PC12 cells and Primary Neurons

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Regulation of Neurite Outgrowths by the Pseudophosphatase MK-STYX in PC12 cells and Primary Neurons

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ABSTRACT

The pseudophosphatase MK-STYX [MAPK (mitogen-activated protein kinase) phosphoserine/threonine/tyrosine binding protein] is a member of the MAP kinase phosphatase (MKP) family. Pseudophosphatases maintain their structure to bind phosphorylated residues, but cannot hydrolyze them; MK-STYX lacks the cysteine and histidine in what would be its catalytic signature motif (HCX_5R). Despite the lack of catalytic activity, MK-STYX plays a role in many cellular signaling pathways such as stress response, apoptosis and neuronal development. Therefore, we wanted to investigate MK-STYX in PC12 neuronal model and primary hippocampal neurons. We hypothesized that MK-STYX overexpression would alter dopamine localization, cytoskeletal protein localization, neuronal extensions and the morphology of primary neurons. The overexpression of MK-STYX increases neurite extension and branching in PC12 neuron-like cells in the presence of nerve growth factor (NGF).
Along with neurite extension and branching, MK-STYX also induces growth cones or actin projections in PC-12 at neurite terminals in the presence of NGF. MK-STYX overexpression also results a similar effect in hippocampal neurons. In our preliminary studies, MK-STYX seems to cause morphological changes when transfected into primary rat neurons. Future studies will focus on exploring the mechanisms behind these morphological changes in neurons. We will also investigate how MK-STYX affects a downstream effector of RhoA, cofilin. Overall, our data provides evidence that MK-STYX causes cytoskeleton rearrangements and validates that pseudophosphatases are important molecules in signal transduction.

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INTRODUCTION

Cells communicate through many mechanisms such as extracellular molecules or cell-to-cell contact. Communication is necessary for proper cell function. When cellular communication fails, cells may fail to grow and differentiation normally and may even die. Reversible phosphorylation of proteins regulates proper communication via signal transduction cascades. Kinases and phosphatases act antagonistically; while kinases catalyze the addition of a phosphate groups, phosphatase catalyze the removal of phosphate groups from various biological molecules. They have the ability to control the rate and duration of response (Tonks, 2006).

Protein tyrosine phosphatases (PTP) are the largest family of phosphatases. They are diversified through alternative promoters, alternative splicing and different post-translational modifications. They remove phosphate groups from phosphotyrosine residues (Tonks, 2006). The catalytic site consists of Histidine-Cystiene-X5-Arginine. The cysteine performs the nucleophilic attack on the phosphorus atom while arginine and an aspartate residue form and stabilize the intermediate structure. A cradle structure forms which allows for amide groups to form hydrogen bonds with the oxygen from the phosphate group (Tabernero et al., 2008). PTPs are divided into the classical phosphotyrosine phosphatases, the dual specificity phosphatases (DUSPs), CDC25-like and low molecular weight phosphatases (Tonks, 2006).

Our research focuses on the dual specificity phosphatases. DUSPs remove phosphate groups from both phosphoserine/threonine and phosphotyrosine residues. DUSPs are important regulators of the mitogen activated protein kinase (MAPK) pathway, which is involved in a myriad of cellular function from apoptosis to differentiation. For example, mitogen activated protein kinase phosphatases (MKPs), a subclass of DUSPs, dephosphorylate threonine and tyrosine on the MAP kinases (MAPKs). MKPs share sequence similarity region (CH2 regions) in

N-terminal domain that allows for effective MKP-MAPK interactions. (Keyse et al., 2002; Tonks, 2006; Wishart et al., 1998)

Pseudophosphatase Members of PTP

Pseudoenzymes are another set of enzymes present in the cell. They differ in their function from prototypical enzymes due to one or more point mutations in the active site that leaves them catalytically inactive. Nonetheless, pseudoenzymes have been shown to perform many other functions in the cell (Tonks, 2009). They have been implicated to act as competitors, spatial anchors, signal integrators and reaction modulators.

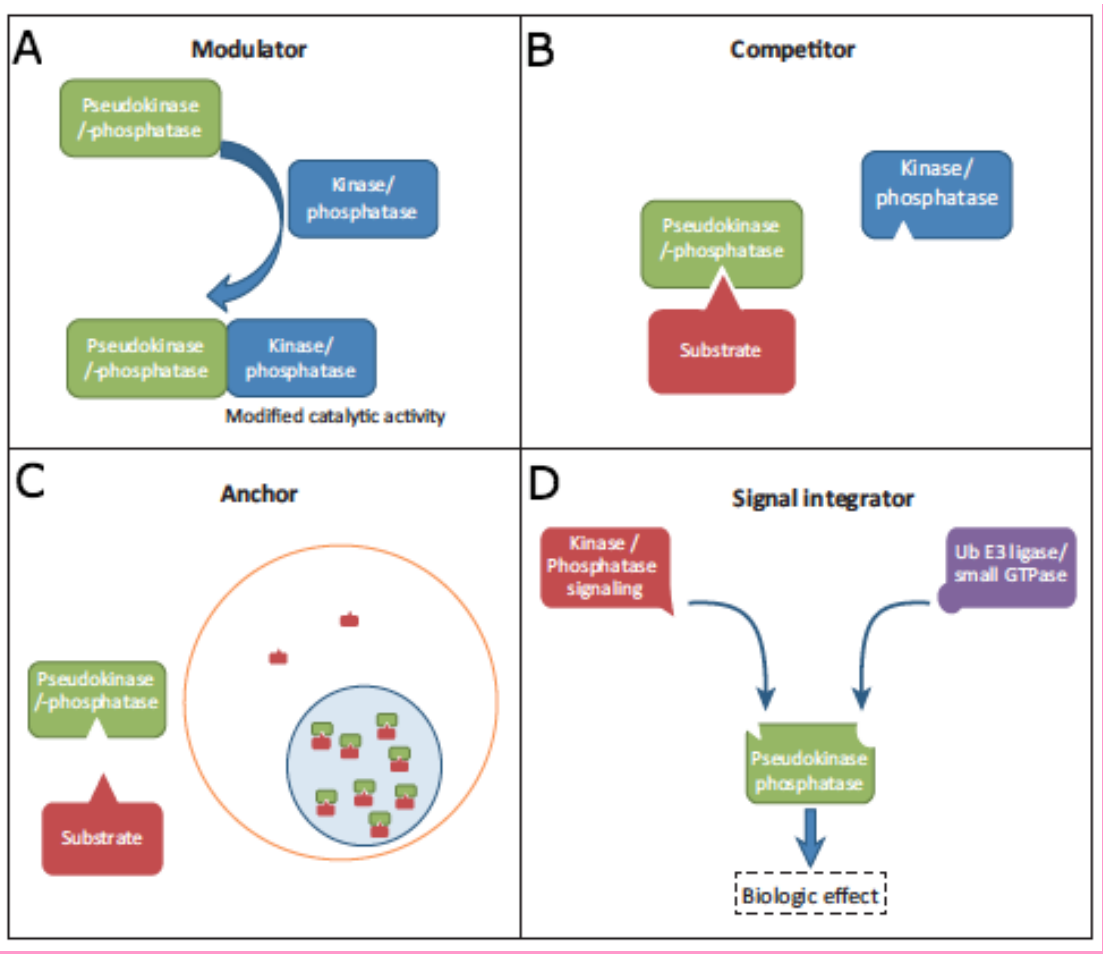

Figure 1. Pseudophosphastases roles in the cell

A) Pseudoenzymes may serve as dimerization partners of kinases or phosphatases, thereby affecting their activities. B) Pseudoenzymes may bind to substrate, acting as competitive inhibitors. C) Pseudoenzymes may bind to substrates and act as subcellular anchor to localize cell components in a specific compartment. D) Pseudoenzymes may also act as integrators by combining signals from different pathways to effect a biological change (Image: Reiterer et al., 2014).

Pseudophosphatase are enzymes that resemble phosphatases but lack critical amino acids in their catalytic site, preventing them from removing phosphate groups from amino acid residues. STYX (phospho-serine, threonine or tyrosine interacting protein), a prototypical pseudophosphatase, contains a glycine residue in place of the nucleophilic cysteine residue that render the protein catalytically inactive. However, this mutation does not interfere with the binding of the pseudophosphatase with the phosphoresidue (Tonks, 2009; Wishart and Dixon, 1998). STYX has been implicated to have numerous functions. In 2015, it was determined that STYX competes with DUSP4 to anchor ERK to the nucleus, thereby affecting Golgi body function (Reiterer et al., 2013). Recently, STYX also has been shown to bind and interact with F-box protein FBXW7. Therefore, STYX prevents FBXW7's binding to the SKP1/CUL1/F-box (SCF) complex and inhibits its tumor suppressor activity (Reiterer et al., 2017).

Other pseudophosphatases may also play important roles in the cell. The dual-specificity pseudophosphatases EGG-4 and EGG-5 trap the dual-specificity tyrosine regulated kinase MBK-2 (mini brain kinase 2) during the oocyte-to-zygote transition in *Caenorhabditis elegans*. Loss of EGG-4/5 results in lethality. Another pseudophosphatase EGG-3 is also involved in controlling MBK-2's access to proteins by sequestering it in the cortex of the oocyte (Cheng et al., 2009; Parry et al., 2009; Tonks, 2009). These findings indicate that pseudophosphatases are involved in

proper development. There are also various pseudophosphatases within the myotubularin (MTM) family of phosphatases that function as scaffolding proteins for proper localization of other proteins. The phosphatase MTMR2 (MTM-related protein 2) interacts with the inactive MTMR13 and certain mutations in either enzyme is associated with 4B Charcot-Marie-Tooth (CMT) disease (Begley and Dixon, 2005; Hinton et al., 2010; Robinson and Dixon, 2005).

These studies point out that pseudophosphatases are not just dead companion of the active phosphatases but rather, are conserved enzymes with their own distinct and important function. This holds true for pseudoenymes in general, as shown in the figure above.

MK-STYX's role in stress granule cycle

Our lab is focused on understanding the cellular function of MK-STYX [MAPK (mitogen-activated protein kinase) phospho-serine/threonine/tyrosine-binding protein], a pseudophosphatase of the MKP family. Histidine and cysteine in the PTP catalytic site are replaced by phenylalanine and serine. Site-directed mutagenesis of the amino acids phenylalanine and serine to their active counterparts histidine and cysteine results in the active mutant MK-STYXactive (Hinton et al, 2010). MK-STYX also has a cdc homology 25 (CH2) domain, which allows for specificity in MKP-MAPK signaling pathways (Wishart and Dixon, 1998).

Lack of the proper amino acids in the active site has not limited MK-STYX's role in the cell. Initial studies revealed that MK-STYX was highly expressed in Ewing's sarcoma family tumors (Siligan et al., 2005). MK-STYX was later shown to complex with G3BP1 [Ras-GAP (GTPase-activating protein) SH3 (Src homology 3)-domain-binding protein1], an RNA binding protein involved in stress granule formation. Stress granules are aggregates of untranslated mRNA, translation machinery and other proteins involved in mRNA translation, which form as a

cellular stress response. G3BP aids in nucleation of the components and can trigger stress granule formation through overexpression or phosphorylation at site serine 149 (Wolozin, 2012; Hinton et al., 2010, Tourriere et al., 2003).

Interestingly, MK-STYX reduced stress granule formation in HeLa cells when it was co-transfected with G3BP1 without affecting G3BP levels. MK-STYX caused similar reduction in stress granules, independent of G3BP1 serine 149 phosphorylation state (Barr et al., 2013; Hinton et al., 2010; Tourriere et al., 2003). MK-STYX affects stress granule formation independent of G3BP1 phosphorylation state, indicating that it must be acting through another pathway. G3BP1 also interacts with RasGAP (Ras GTPase activating protein), a regulator of Ras GTPase subfamily. The subfamily is part of the MAPK/ERK signal transduction cascade, which is initiated by growth factors important in neuronal cell lines (Irvine et al., 2004).

The effects of MK-STYX on PC12 Cells

PC12 cells are extensive neuronal cellular model systems derived from rat pheochromocytoma cells from the adrenal medulla. They have an embryonic neural crest origin, which allows them to differentiate into sympathetic-like neurons upon stimulation by nerve growth factor (NGF) stimulation (Greene and Tischler, 1976). Neurotrophins like NGF, brain derived neurotrophic factor, neutrophin-3 activate intracellular signaling cascades by binding to tyrosine receptor kinases (Trk). NGF binds to a tyrosine receptor kinase A (TrkA), activating MAPK/ERK pathway as well as the RhoA pathway (Klesse et al., 1999). NGF causes a sustained activation of the MAPK/ERK pathway and PC12 cells cease division and differentiate into neuron-like cells. Additionally, upon NGF stimulation, MAPK is translocated from the cytoplasm to the nucleus. On the other hand, epidermal growth factor (EGF) only transiently

activates the pathway, resulting in proliferation and no MAPK translocation (Traverse et al., 1992).

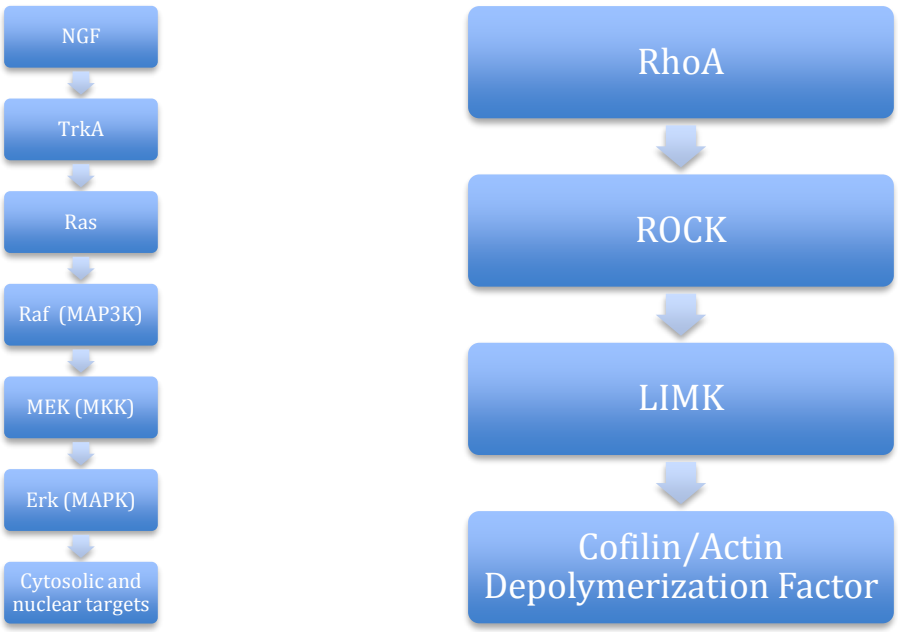

Figure 2. PC12 differentiate through different pathways

Nerve growth factor binds to cell surface tyrosine receptor kinase A (TrkA), inducing dimerization and transphosphorylation. Its activation leads to activation of its downstream adaptors, such as Src homology-2 (SH2) and Son of Sevenless (SOS), which go on to activate Ras GTPase. Ras is a small GTPase that switches between an active GTP bound state and an inactive GDP bound state with assistance from guanine exchange factor (GEF) and GTPase activating protein (GAP). Its activation activates the conserved MAPK pathway via phosphorylation. Extracellular signal-regulated kinase (ERK) goes on to phosphorylate and activate cytosolic and nuclear targets like transcription factors and ribosomal protein kinases (Klesse et al., 1999).

The Rho superfamily, which consists of small GTPases like RhoA, Rac1 and Cdc42, is part of the Ras superfamily. These small GTPases have been implicated in cytoskeletal reorganization and cell morphology. RhoA is activated by chemorepellants and results in growth cone collapse, cell rounding and a decrease in cell adhesion to the extracellular matrix. Rac1 is involved in membrane ruffling and lamellipodia and growth cone formation and Cdc42 induces filopodia formation (Kozma et al., 1997). NGF stimulation activates Rac1 and recruits it to protrusion sites with F-actin. Additionally, NGF activates protein kinase A, which phosphorylates RhoA at serine 188 and renders it unable to bind to its downstream effector, Rho associated kinase (ROCK) (Nusser et al., 2006). Rac1 and RhoA exert antagonistic effects on each other: activated Rac1 disables RhoA from activating ROCK whereas RhoA induces a down-regulation of Rac1 activation via ROCK (Yamguchi et al., 2001). ROCK phosphorylates its downstream effort, LIMK, which phosphorylates the actin depolymerization factor family (Huang et al., 2006).

STYX has also been implicated in playing a role in these neuronal progenitor cells. STYX competes with MKP-2 to bind to ERK, ultimately disabling PC12 cells from differentiating (Wishart & Dixon, 1998). Interestingly, our lab has shown that MK-STYX has the opposite effect on PC12 cells: differentiation and extension (Flowers et al., 2014). Seventy-two hours post-NGF stimulation, MK-STYX transfected cells were expressing more and longer extensions. Initially, we investigated whether the extensions were occurring through the MAPK pathway, since MK-STYX resembled MKPs. When MAPK kinase (MEK) was inhibited, the

cells produced these extensions. Therefore, we investigated the role of the RhoA pathway for this phenotype. As mentioned before, active RhoA causes cell rounding and decreases neuronal extensions. Therefore, we determined the activity of RhoA in the presence of MK-STYX using an activity assay. Compared to control cells, activity of RhoA was reduced in MK-STYX transfected cells, indicating that the RhoA pathway affects these extensions (Flowers et al., 2014). Overall, MK-STYX seems to be affecting PC12 differentiation via the RhoA pathway and not the MAPK pathway.

Actin and Cofilin Roles in Differentiation

In order to understand MK-STYX's effects in the cell, it is important to look at the downstream effectors of RhoA including a protein called actin. Actin is a ubiquitous and conserved eukaryotic protein important in many cellular functions such as cellular motility, cytoskeletal rearrangement and the cell cycle.

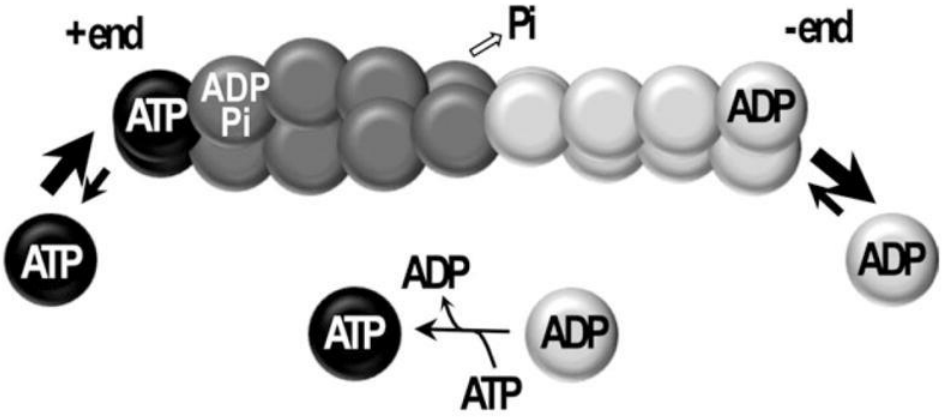

Figure 3. Actin monomers undergo treadmilling

Actin monomers are bound to adenosine triphosphate (ATP) and a divalent cation. The nucleotide is then hydrolyzed to ADP and inorganic phosphate by an internal ATPase activity. The ATP-actin monomers that have not undergone hydrolysis are at the plus end of the filament (barbed end) where as the ADP-actin monomers make up the minus end (pointed end). In many cells, actin may fan out and create a veil-like network called a lamellipodia or create bundled micro-projections called filopodia (Cooper, 2000).

Cofilin/actin depolymerizing factor (ADF) family of proteins regulates actin filament assembly. Its function is phosphoregulatory: When it is dephosphorylated, cofilin induces a conformational twist in the actin filament on the minus side, leading to release of actin monomers (Meberg et al. 2002). It removes actin monomers from the minus, rear of filamentous actin (F-actin) and moves the monomers to the leading edge of the filament, allowing for growth or movement in one direction. When cofilin is phosphorylated at N-terminal serine 3, cofilin loses its ability to bind actin monomers and filamentous actin is stable. The stable filamentous actin maintains the structure of the cell at that location. Cofilin works in conjunction with a branching enzyme complex called Arp 2/3 (actin related protein), which preferentially allows actin branching from cofilin-severed F-actin filaments (Huang, 2006).

Cofilin is phosphorylated at Ser3 residue by the TES (testicular protein) kinases and the ubiquitous LIMK. Whereas TESK is activated by integrin attachment, LIMK is activated through a variety of pathways including Rac/Cdc42 pathway and the previously discussed RhoA pathway. Dephosphorylation of cofilin is mediated by SSH (slingshot), CIN (chronophin) and protein phosphatase 1 and 2A (Huang, 2006; Tsai and Lee, 2012).

Slingshot

Slingshot is a conserved protein that is distantly related to MAP kinase phosphatases (Huang, 2006). Loss of slingshot resulted in excess actin protrusions in Drosophila (Niwa, Ohashi, 2002). It has a conserved A and B domain and a PTP domain and sometimes a C-

terminal F-actin-binding domain (Soosairajah et al., 2005). However, slingshot has substrate specificity for cofilin while other phosphatases like MKP-5 were able to decrease cofilin phosphorylation in Cos7 cells (Niwa et al., 2002). There are three known human isoforms of slingshot, which are all widespread in the body but differ in subcellular localization and activity patterns (Huang, 2006l Soosairaja et al., 2005).

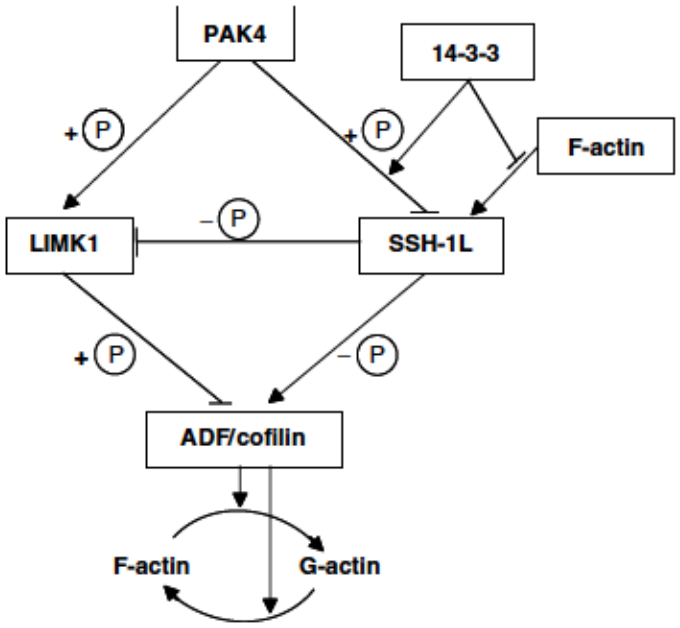

Figure 4. Cofilin is phosphoregulated

In E18 rat hippocampal cells, endogenous LIMK1, SSH-1L and 14-3-3 scaffolding proteins are co-localized in growth cones, with the strongest association between SSH-1L, 14-3-3 and actin. 14-3-3 proteins are highly conserved proteins that are important in many cellular processes such as cell cycle regulation and the stress response. Although they lack a functionally catalytic domain, they have a binding domain for phosphorserine/threonine residues. This binding may change the localization, the interactions or the activity of the target protein, similar to the proposed modes of action of pseudoenzymes (Tzivion, 2001). LIMK1 uses its kinase domain to associate with SSH whereas SSH uses its A, B and phosphatase domain. This interaction suggests a more regulatory function for either LIMK-1 or SSH-1L. LIMK-1 is first activated by phosphorylation at Thr508, followed by autophosphorylation at other residues. SSH-1L functions to remove phosphate groups from these sites, reducing LIMK-1's action on cofilin (Image: Soosairajah et al., 2005).

Slingshot is also phosphoregulated by PAK4 (p21 activated kinase 4), one of the kinases that activate LIMK1. Phosphorylation of SSH-1L resulted in higher levels of phosphorylated LIMK compared to LIMK-1 with unphosphorylated SSH-1L. Consequently, phospho-cofilin levels were also reduced under unphosphorylated SSH-1L (Soosairajah et al., 2005). Dephosphorylation of slingshot by the phosphatase calcineurin has been shown to enhance its function. Additionally, F-actin may also bind to slingshot to increase its phosphatase activity on cofilin (Huang et al., 2006). Slingshot is also directly phosphorylated by a serine/threonine kinase, protein kinase D (PKD).

The phosphorylation state of slingshot affects its interactions. Slingshot's association with 14-3-3 scaffolding protein is dependent on its phosphorylation state at serine 937 and 978

(Peterburs et al., 2009). Binding of 14-3-3 reduces binding of slingshot to F-actin, reducing slingshot's activity (Soosairajah et al., 2005). 14-3-3 scaffolding protein sequesters slingshot in the cytoplasm. Upon release, slingshot is activated and localizes to cellular extensions (Huang et al., 2006). The localization and interaction of all these proteins indicate that the dephosphorylation of slingshot occurs in the cytoplasm, whereas the inactivation via phosphorylation occurs in the extensions (Peterburs et al., 2009).

Axonal and Dendritic Growth

Because of the effects that were previously observed in PC12 cells, looking at the effect of MK-STYX in primary neurons is also an avenue to explore. Primary neurons have distinct (but somewhat similar) characteristics from PC12 cells. Primary neurons have axons and dendrites originating from the cell body. While the axon is mainly responsible for sending messages to other cells, the dendrite receives these messages from other axons across the synaptic cleft. When PC12 cells are differentiating, they produce extensions that resemble minor processes of primary neurons. Because they are progenitor cells, they can't form axons and dendrites simultaneously as neurons do (Das et al., 2004; Jeon et al., 2010).

Axons and dendrites get their shapes from microtubule filaments. The plus end of the microtubule is where the beta-tubulin subunit is exposed, whereas the negative end of the microtubule is where alpha-tubulin is exposed. This positioning of the different subunits allows the microtubule protofilament to have polarity. While both dendrites and axons are strengthened by tubulin, the polarization of the microtubules is different in each. In axons, the plus ends are directed towards the distal ends of the extensions whereas for dendrites, microtubules can be polarized in either direction (Bouquet and Nothias, 2007). Microtubules are stabilized by structural microtubule-associated protein (MAP) like MAP2 and Tau. While MAP2 is associated

with dendrites, Tau is associated with axons and tends to associate with their sub-cellular regions after the extensions show some resemblance to their future functional morphology (Kosik 1987). Previous research has indicated that PC12 cells form synapse-like connections between Tau-enriched extensions and MAP2-enriched extensions (Jeon, 2010). Additionally, under NGF stimulation, the amount of MAP like tau and MAP1 increased and are considered limiting factors to neurite extensions. This means that these proteins, not tubulin accumulation, are necessary for the outgrowths (Drubin, 1985).

Newly plated embryonic cells in culture undergo five developmental stages to become mature neurons. Initially, they send out minor processors from which one starts to develop as the axonal extension. This extension has a dynamic growth cone, high actin turnover and actin filament destabilization (Dotti, 1988). The mammalian tumor suppressor kinase LKB1 kinase is also localized to the axonal extension and phosphorylates SAD (Synapses of the Amphid Defective) kinases. SAD kinases in turn phosphorylate axonal microtubule-associated proteins like Tau (Kim et al., 2010; Shelly et al., 2007).

The chemoaffinity hypothesis is another way to understand how specific and accurate connections are formed between axons and dendrites. Based on initial experiments by John Langley and Roger Sperry, there are currently three models proposed for this hypothesis. The first is that a gradient of chemoattractants and repellants that guide a growing axon towards its target. Axons may also be guided by a combination of contact and diffusible molecules for synaptic specificity. Finally, neurons may be expressing differential adhesion molecules, such as the G-Protein coupled receptor (GPCR) in olfactory neurons that allow for cell-to-cell specificity.

Down Syndrome cell adhesion molecules (dsCAMs) in insects and its vertebrate homologue, protocadherin (Pcdhs) have been shown to specify neuronal assembly via a hemophilic binding and self-avoidance repulsion mechanism (Weiner et al., 2013). A neuron expresses the same isoform of dsCAM along its extensions. Homophilic binding between these identical dsCAMs results in self-recognition and repulsion between the branches. Cells expressing protocadherins utilize a similar isoform-specific hemophilic binding mechanism. Additionally, vertebrate neurons lacking Pcdhs have neuronal projection error and undergo rapid apoptosis. These molecules point to the fact that neurons have mechanisms to prevent overlap and self-crossing of their branches (Zipursky, 2010).

Cofilin & growth cones roles in axon guidance

Growth cones are mobile tips of the growing axon that is responsible for steering the axon towards or away from a stimulus. They are characterized by a microtubule-rich central domain and an actin-rich peripheral domain, which include both lamellipodia and filopodia.

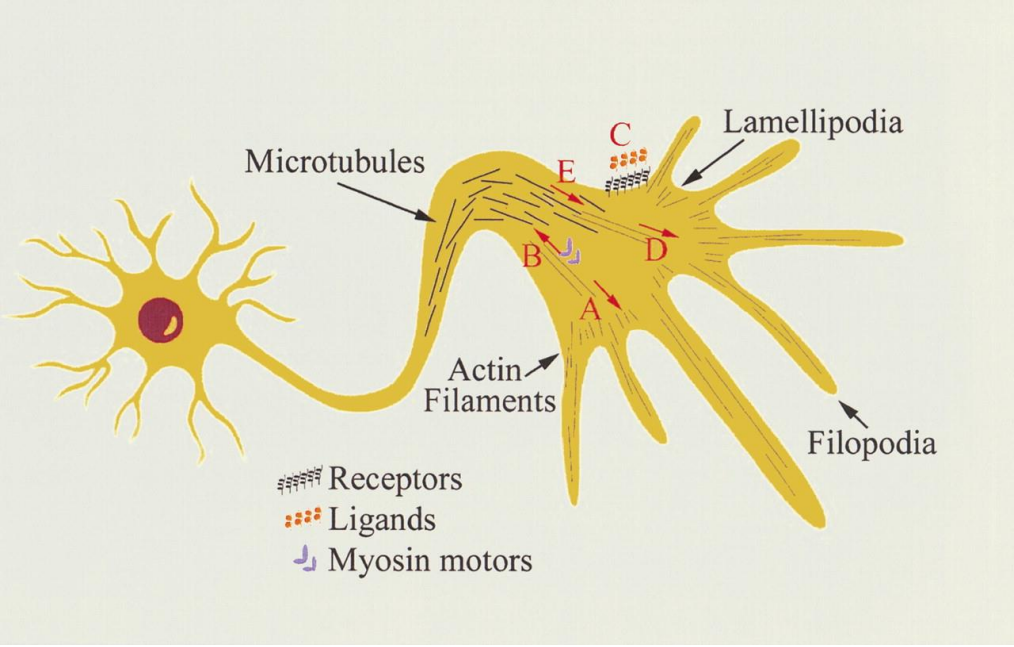

Figure 5. Growth cones form by cytoskeletal interactions

Growth cones are directed by a complex interplay of microtubules, actin and myosin-like motors. A) Actin monomers polymerize into filaments towards the leading edge of the axon. B) Myosin-like motors drag actin filament towards the central domain where depolymerization occurs. Microtubules remain in the central domain due to retrograde flow. The balance between actin polymerization and retrograde flow determines rate of axonal growth or retraction. C) Ligands (like cell-adhesion molecules) may bind to surface receptors. D) A positive signal from the binding is transferred to the cytoskeleton via other proteins and signaling cascades resulting in anterograde flow of actin. E) Microtubules also move into the peripheral domain, elongating the axon towards the stimuli in this case (Goldberg, 2003).

Growth cones may form by growth cones branching where one growth cone splits off into two or more cones or may form by interstitial branching where a growth cone extends from the trunk of an extension. Other cytoskeletal proteins like MAP2, Tau and dynein also affect the stability of growth cones.

Thesis Objectives

Previously, our lab has shown that MK-STYX induces neurite-like outgrowths in PC12 cells and knockdown of MK-STYX reduces these outgrowths. Additionally, we showed that MK-STYX reduces RhoA activity in the first 24 hours after NGF stimulation, stabilizes extensions with microtubule stabilizing proteins such as MAP2 and Tau and increases growth cone formation. Therefore, we wanted to further our understanding of the relationship between MK-STYX and neurite extensions by answering the following questions:

- How does MK-STYX affect the localization of dopamine and slingshot?
- How does MK-STYX change the internal cytoskeleton of PC12 cells?
- Does MK-STYX affect neuronal extensions, growth and survival?
- Does MK-STYX affects primary hippocampal neurons?

METHODS

Plasmids

pMT2-FLAG-MK-STYX-FLAG and pMT2-FLAG-MK-STYXactive-FLAG were generated as described by Hinton *et al*. 2010. The constructs were flanked by FLAG epitope. FLAG-GFP-MK-STYX-FLAG and FLAG-mCherry-MK-STYX-FLAG plasmids were created by Vincent Roggero.

PC12

Cell Culture

PC12 cells were maintained at 37 °C in 5% CO_2 in Roswell Memorial Institute (RPMI) medium (Invitrogen) supplemented with 10% horse serum (Invitrogen) and 5% fetal bovine serum (Invitrogen). Cells were serum starved using Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen) supplemented with 0.1% FBS serum starve medium. Cells were maintained with 15 ml medium per 75cm^2 flask (Thermo Scientific), 5 ml per 15cm^2 flask (Fisher), 5 ml per 60 mm plate (Thermo Scientific), or 3 ml per well in six well plates (Thermo Scientific). Cells were grown on collagen-covered slips in six well plates.

Transfection

Twelve to eighteen hours post seeding, transient transfections were performed using Lipofectamine 2000 (Invitrogen). For transient transfections, pMT2 control vector, pEGFP control vector, mCherry control vector, pMT2-FLAG-MK-STYX-FLAG, pMT2-FLAG-MK-STYXactive-FLAG, FLAG-GFP-MK-STYX-FLAG or FLAG-mCherry-MK-STYX-FLAG were used. Cells were transfected using 2 μl of Lipofectamine 2000 reagent per 1 μg DNA using serum-free Opti-MEM (Invitrogen). Cells were incubated with transfection reagents for four to

six hours before the medium was removed and replaced with fresh medium. Four hours after the addition of the new medium, the cells were serum starved for about 12 hours.

NGF Stimulation

Twenty-four hours post-transfection, PC12 cells were serum starved in DMEM supplemented with 0.1% fetal bovine serum for eight to twelve hours. PC12 cells were then stimulated with 100 ng/ml of b-Nerve Growth Factor (Prospec).

Time-dependent NGF Stimulation

PC12 cells were transfected, serum starved, and stimulated with NGF as described for time points consisting of 0 minutes, 3 minutes, 5 minutes, 12 minutes, 30 minutes, 24 hours, 48 hours, or 72 hours. Cells were lysed at the indicated time points.

Immunostaining

Cells were grown in six-well plates at a confluency of 2 X 10^4 cells on Type 1 collagen-coated coverslips (Neuvitro). 72 hours post-NGF stimulation, cells were prefixed in 3.7% percent formaldehyde for five minutes. Following prefixation, cells were fixed in 3.7% formaldehyde to cross-link protein in the extracellular matrix for 10 minutes. The cells were then permeablized by 0.1% Triton-X-100 for 5 minutes. Cells were then probed with a primary antibody solution for an hour in a humidifiying chamber. The solution contained the antibody at a certain concentration and goat serum (Santa Cruz Biotech) diluted in D-PBS with $CaCl_2$ and $MgCl_2$. Following the staining, the cells were probed with a fluorescently tagged secondary antibody for 45 minutes. Cells were then washed, mounted on glass slides with FluroGel with DAPI (Electron Microscopy Science).

Fluorescence Microscopy

Nikon Eclipse Ti inverted fluorescence microscope was used for phase and fluorescence live microscopy of PC12 cells and primary neurons.

Quantification of primary neurites

PC12 cells were grown on 6-well plates on collagen-covered coverslips at a confluency of 2 X 10^4 cells Cells were transfected with 2 μg pEGFP control vector and FLAG-GFP-MK-STYX-FLAG MEM (Invitrogen) and stimulated with NGF for seventy-two hours.

In order to quantify data on neurite extensions and branching of PC12 cells under different plasmid conditions, cells were scored for primary neurites. These were defined as outgrowths originating from the cell body that were at least 20 μm in length. Measurements were made using NIS-Elements BR 3.10 software (Nikon).

Primary Neurons

Cell Culture

E18 Sprague Dawley rat hippocampal neurons (Brainbits) were spun down at 1100 rpm after delivery for 1 minute and the supernatant discarded. The cells were resuspended in 1 ml NbActiv1 media (Brainbits), counted and plated for standard transfection in a six-well plate with poly-D-lysine coverslips for optimal neuronal attachment. The neurons were maintained at 37 °C in 5% CO_2 with 3 mL media per well.

Transfection

Neurons were transiently transfected using standard PC12 transfection protocol with Lipofectamine 2000 (Invitrogen) twelve to eighteen hours post seeding, as mentioned above. Cells were transfected using 2 μl of Lipofectamine 2000 reagent per 1 μg DNA using

Transfection Medium (Brainbits). Neurons were transfected with FLAG-GFP-MK-STYX-FLAG or GFP control vector.

Electroporation

The neurons were also transfected using the electroporation technique. After counting cells, approximately 100,000 neurons were spun down and the media removed. The cells were suspended in 100 µl of intracellular buffer INB (135 mM KCl, 0.2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 5 mM EGTA, pH 7.3), which results in about 3 nM free calcium.buffer was added to the cells. and the cells were pipetted into an electroporation cuvette. 2 µg plasmids were added to the solution. The cuvette was inserted into the electroporator (Bio-rad) and the voltage was set to 300 mVolts. The electroporator button was pressed until there was a beep from the machine, indicating the end of the electroporation. The actual voltage delivered and time constant was recorded. The cells were then transferred from the cuvette to a well in a 6-well dish with PDL-coated coverslips. Three mL of NbActiv media was added to the well.

Staining

Three to four days after seeding the cells, the coverslips were mounted on glass slides with FluroGel with DAPI and visualized under the fluorescence microscope to see the expression and localization of the proteins under electroporation and standard transfection conditions.

RESULTS

MK-STYX affects PC12 differentiation and neurite extensions under NGF stimulation

Previous work in our lab has shown that MK-STYX induces significantly more primary and secondary neurites in PC12 cells, compared to our positive and negative controls. These extensions are especially prominent under NGF stimulation. When shRNAs against MK-STYX was used in NGF-stimulated cells, the cells developed less neurite extensions and displayed a more rounded morphology (Flowers et al., 2014; Dallas Banks 15' unpublished data).

A follow-up study was conducted with new plasmid vectors. PC12 cells were transfected with either pEGFP or GFP-MK-STYX. Twenty-four hours post-transfection, the cells were stimulated with 100 ng/ml NGF. Seventy-two hour post-stimulation cells were imaged for primary neurites (extensions from cell body) ≥20 mm, and representative images were taken. We found that cells expressing MK-STYX had a higher percentage of cells with one or more extensions as compared to the cells expressing the pEGFP vector. Additionally, at the extreme end of the spectrum, cells with MK-STYX showed significantly higher percentage of cells with four or more neurites (Figure 6).

MK-STYX affects MAP2 and Tau localization

We wanted to examine the distribution of dendrite-like extensions and axon-like extensions in PC12 cells with or without MK-STYX overexpression. We stained for axonal (pre-synaptic) marker Tau and dendritic (post-synaptic) marker MAP2. PC12 were transfected with control vector pMT2 or MK-STYX and either stimulated or not with NGF. Seventy-two hours post-transfection, cells were fixed, permeabilized and stained with MAP2 conjugated to FITC antibody and Tau conjugated to Cy3 antibodies. Slides were visualized to study the localization of the two proteins.

MAP-2 was localized in the cell body in non-stimulated cells, but also in major processes in stimulated cells or cells over-expressing MK-STYX. Tau was localized to the cell body in non-stimulated cells, whereas it was more intense in neurites in NGF stimulated cells or MK-STYX expressing cells. Their localization in the major processes indicates that stimulated control cells and MK-STYX expressing cells have pre-synaptic (axons-Tau) and post-synaptic (dendrites-MAP2) processes. The connections between the presynaptic and post-synaptic cells also seem to be enhanced in cells expressing MK-STYX and stimulated with NGF compared to the control cells (Figure 7, Figure 8, Figure 9).

MK-STYX affects actin and tubulin localization

To understand how the expression of cytoskeletal proteins was affected by MK-STYX, we decided to fluorescently tag and observe the localization of actin and tubulin. PC12 cells were transfected with pMT2 control vector and MK-STYX, then NGF stimulated and allowed to differentiate or divide for 72 hours. Cells were fixed in formaldehyde, permeabilized by trypsin and immunostained with rhodamine-conjugated antibody for actin and anti-B tubulin conjugated to FITC for tubulin in microtubules. Under pMT2 in the absence of NGF, both actin and tubulin were uniformly distributed in the cell body. For MK-STYX cells without NGF stimulation, actin and tubulin were distributed in the cell body and along the neurites (Figure 10).

Similarly, in MK-STYX cells stimulated with NGF, both actin and tubulin were distributed in the cell body and along the neurite extensions. Actin also seemed to localize strongly in bundles at the end of the neurites. These bundles were present not only at the neurite tips but also along the microtubule-rich neurite. For control cells stimulated with NGF, actin was primarily localized to the cell body and extended into the beginning portions of neurites (Figure 10).

MK-STYX induces more growth cones under NGF stimulation

These actin protusions along and at the ends of neurites are indicative of growth cones, dynamic structures that guide growing neurites. To better examine these growth cones, we took magnified images of neurite shaft in NGF stimulated control and MK-STYX cells. Veil-like Actin-rich protusions were observed at the ends of neurites and along the neurite shaft mostly for cells overexpressing MK-STYX and stimulated with NGF (Figure 11). These veil-like protusions are indicative of lamellipodia of growth cones and may be important for extensions to reach their synaptic partners (Cooper, 2000).

To quantify the number of growth cones in the different samples, we initially blinded the slide set (covered the identifying information about each slide) so there would be no researcher bias. We then looked at 100 cells from each sample and manually counted the percent of cells that had growth cones. The cells chosen were located all over the slide. We found that the cells overexpressing MK-STYX and stimulated with NGF showed significantly more growth cones compared to control cells stimulated with NGF. An unpaired t-test was performed between the two sets, which gave a p-value of $< .05$ (Figure 12).

Something interesting we noted is that the percent of cells with growth cones was significantly lower for cells not stimulated with NGF and expressing MK-STYX compared to control cells not stimulated with NGF. This difference under no NGF stimulates perhaps indicates that MK-STYX's activity is dependent on the activation of NGF pathways.

MK-STYX does not affect dopamine localization

PC12 cells release dopamine and norepinephrine their neurotransmitters for communication (Greene and Tischler, 1976). We wanted to examine whether dopamine would cluster at the ends of the neurites and whether localization would be different under MK-STYX

overexpression. PC12 cells were seeded, transfected with GFP-MK-STYX or pEGFP control vector, then either stimulated with NGF or not, and allowed to differentiate for 72 hours. Cells were then pre-fixed and fixed in formaldehyde, permeabilized by trypsin and stained with dopamine primary/α-rabbit Cy3 secondary antibodies. Slides were visualized under phase and fluorescence microscopy to examine dopamine localization.

Dopamine was uniformly distributed in the cytoplasm for all the conditions observed. There was no noticeable difference observed in the cells with the control vector or GFP-MK-STYX overexpression (Figure 13).

MK-STYX does not affect phospho-slingshot localization

As mentioned before, cofilin is dephosphorylated by slingshot. Slingshot's activity is also dependent on its phosphorylation state, which dictates its localization within the cell. If slingshot is phosphorylated, it is sequestered in the cytoplasm by 14-3-3 scaffolding protein whereas if it is dephosphorylated, it is localized to the extensions (Nagata-Ohashi et al., 2004).

To determine whether the localization of phosphor-slingshot changed, PC12 were transfected with pMT2 or MK-STYX, stimulated or not with NGF for 72 hours. Cells were then fixed, permeabilized and stained for phospho-slingshot primary/ α-rabbit Cy3 secondary antibodies. Cells were then visualized using phase and fluorescence microscopy. With NGF stimulation, phosphor-slingshot was localizing more to the cytoplasm. However, there wasn't much of a difference in localization between the MK-STYX overexpression and control cells (Figure 14).

MK-STYX changes survival patterns of neurons

MK-STYX induces morphological and cytoskeletal changes in PC12 cells. Although they are useful and important model systems, we wanted to observe primary neurons to see how the

effects of MK-STYX differ between PC12 cells and neurons. Embryonic hippocampal cells were electroporated with pMT2 control and MK-STYX and grown on PDL-coated coverslips in 6-well plates in NbActiv media. Cells were visualized using phase and fluorescence microscopy.

The two different culture conditions (control or MK-STYX transfection) were remarkably different from one another. The cells expressing pMT2 showed individual cells largely separated from one another and forming one or two connections to other neurons. Cells expressing MK-STYX, however, were often growing in clumps and had a web-like network of connections through the culture. There were also a lot more cells observed per area in MK-STYX cells compared to the control cells. Additionally, cultures with MK-STYX showed a lot more debris compared to the control cells (Figure 15). The debris and the large number of neurons in the culture for MK-STYX may validate the previous research that MK-STYX is important in the cellular survival and apoptosis (Nieme et al., 2011; Nieme et al., 2014).

MK-STYX induces more primary outgrowths in neurons

Previous research and work shown here has shown that MK-STYX induces more primary and secondary outgrowths from PC12 cells. We wanted to see if MK-STYX overexpression induced a similar change in the cell morphology of primary neurons. Rat hippocampal neurons were co-electroporated with pEGFP and pMT2 as our control and pEGFP and MK-STYX. They were then seeded in six-well plates on PDL-coated coverslips with NbActiv media. After three to four days hours, cells were imaged using phase and fluorescence microscopy. In cells expressing the control vector, we frequently observed one long neurite (presumbably the axon) and a few other extensions coming from the soma. In cells expressing MK-STYX, it was harder to distinguish the axon from the other extensions. Additionally, there were significantly more extensions originating from the cell body as compared to the control (Figure 16).

Something we noticed in these experiments was that cells expressing MK-STYX had neurites from the same cell overlapping and intertwining with each other. Normal neurons express adhesion molecules that participate in hemophilic binding and self-avoidance repulsion mechanism to prevent extensions from the same neuron from sticking together (Zipursky, 2010). This unusual behavior of neurites in MK-STYX cells may be indicative of deregulation of pathways and proteins caused by overexpression of MK-STYX.

MK-STYX localizes to the ends of neuronal extensions

In order to study the localization of overexpressed MK-STYX in rat hippocampal neurons, neurons were co-transfected with pEGFP and pMT2 as our control and pEGFP and MK-STYX. The cells were initially mixed with IBN buffer and the plasmids were electroporated into the cells. Following electroporation, cells were plated on PDL-coated coverslips in six-well plates with NbActiv media.

Neurons were imaged using phase and fluorescence microscopy after three- four day. In cells expressing pMT2 control vector and pEGFP, the fluorescence is localized mainly to the cell body. However, in cells expressing MK-STYX and pEGFP, the fluorescence is present not only in the cell body but along the length of the longest extension (presumably the axon) (Figure 17). This localization of the protein along the neurite may be indicative of the type of interactions MK-STYX is having with other cellular components.

DISCUSSION

Summary

Our research has shown that MK-STYX may play a critical role in neurite formation, branching and movement, cytoskeletal rearrangement and phosphoregulatory pathways in PC12 cells. MK-STYX induces more primary neurites and secondary neurites and affects localization of axonal marker Tau and dendritic marker MAP2. It also affects tubulin and actin localization and induces more growth cones. Furthermore, we found that MK-STYX induces more primary extensions and changes cell survival patterns in rat hippocampal neurons. Observing these changes in neurons validates and confirms our neurite extension results from the PC12 model system.

By examining MK-STYX's mechanisms, we may be able to effectively design therapies to treat neurodegenerative diseases like Alzheimer's. Amyloid-beta plaques and neurofibrillary tangles caused by hyperphosphorylated tau lead to loss of connections between neurons, decreasing cell-to-cell synaptic communication (Ittner, 2010; Morris, 2011) If MK-STYX is able to induce neurite extensions, affect the localization of proteins like tau and significantly reorganize the cytoskeleton, it seems to be a viable therapeutic candidate to explore in the future. Furthermore, previous work in our lab has shown that aggregation of proteins like tau may increase the formation of stress granules, which in turn increase aggregates. MK-STYX has been shown to reduce stress granule formation in HeLa cells and colocalize with some aggresomes (Barr et al., 2013; Roni Nagle 16', unpublished).

PC12 cell neurite outgrowths

PC12 cells are neuronal model systems derived from rat adrenal medulla. Upon stimulation by a neurotrophin like NGF or sustained activation of the MAPK pathway, PC12

cells undergo differentiation to become neuron-like cells capable of transmitting neurotransmitters like dopamine. On the other hand, upon stimulation by epidermal growth factor (EGF) or transient activation of the MAPK pathway, PC12 cells don't differentiate and continue to divide.

Previous work in our lab has shown that overexpression of the pseudophosphatase MK-STYX induces neurite outgrowths, enhances the effect of NGF and is required for NGF-induced neurites compared to the control (Flowers et al. 2014). We wanted to examine whether there was a change in the number of extensions coming from the cell body (primary neurites) and the number of secondary extensions branching from the neurites (secondary neurites). This would allow us to quantify the distribution of neurites under different plasmid conditions. Previous work showed that in the absence and presence of NGF, MK-STYX caused a significant shift in the distribution of cells containing one, two, or three neurites compared to the control (Dallas Banks 15'). These results were validated and supported by work conducted in this thesis regarding neurite extensions with MK-STYX conjugated with a fluorescent protein marker.

In order for these extensions to be physiologically functional, they have to meet several requirements. They need to cover an area that encompasses its synaptic input, branch sufficiently and have a dynamic cytoskeleton for growth and movement (Wassle and Boycott, 1991). Based on our previous and current results, the neurites fulfill all of the requirements. MK-STYX induces longer neurites (at least ≥20 mm) compared to the control, a larger distribution of more primary and secondary neurites and more growth cones per cell under NGF stimulation.

Given that MK-STYX induces these changes in PC12 cellular morphology, it must play a role in establishing axonal and dendritic connections and cytoskeletal reorganization.

PC12 cytoskeletal regulation

The next step was to understand the pathway by which MK-STYX may be causing these morphological changes in the cell. The cellular cytoskeleton is a dynamic body composed of many proteins and involved in many functions, including neurite extension in neuronal cells.

MK-STYX decreased the activity of RhoA, a cytoskeletal rearrangement protein, compared to the control. Inactivation of RhoA is essential for neurite outgrowths. MK-STYX also affected phosphorylation patterns of cofilin, an actin-depolymerizing factor and a downstream effector of RhoA (Flowers et al., 2014). Taken together, these results provide evidence of MK-STYX's effects on neurite outgrowths and the interactions it might have to cause these effects. Because Rac1 and RhoA have antagonistic roles, we wonder whether MK-STYX is activating Rac1 to not only inactivate RhoA but also to induce growth cones (Yamaguchi et al., 2001).

Growth cones are dynamic structures at the distal ends of neurites that respond to extracellular cues. They are made up of cytoskeletal proteins like actin and tubulin whose interactions lead to period of growth and periods of retraction. Growth cones arise from two different mechanisms: growth cone splitting where growth cones split off from the end of a neurite or interstitial branching where a growth cone arises along a neurite shaft (Lewis et al., 2013). MK-STYX causes changes in the localization and arrangement of tubulin and actin. In control cells, actin is mainly localized at the beginning of the neurite extensions. Cells overexpressing MK-STYX showed actin localization all along the neurite and formation of small bundles of actin at the tips, which is characteristic of growth cones. Under NGF stimulation, cultures overexpressing MK-STYX had significantly more cells with growth cones than control cells. It is interesting to note that without NGF stimulation, the control culture had more cells with growth cones than the culture expressing MK-STYX. There may be numerous reasons for

this. NGF activates Rac1 and Cdc42, which are important in lamellipodia, filopodia and growth cone formation (Kozma, 1997). If MK-STYX is acting along these pathways, the NGF stimulation may be necessary for MK-STYX to have an effect. MK-STYX may also be having an inhibitory effect on growth cone formation without NGF stimulation. Further studies will need to be conducted to verify and identify interacting partners.

Because of the dramatic changes to the internal cytoskeleton, we questioned whether MK-STYX would also change neurite connections and synaptic transmission. PC12 cells' neurites with a high concentration of the axonal marker Tau-1 connect to neurites with a high concentration of the dendritic marker MAP2 (Jeon, 2010). Neurites from one cell to another were forming under both conditions but the connections seem to be enhanced under MK-STYX overexpression.

Recent studies have also shown that PC12 cells release norepinephrine and dopamine but the level per cell of dopamine does not change for NGF-stimulated cells compared to control PC12 cells (Greene and Tischler, 1976). Our immunostaining also seem to indicate that the levels of dopamine do not change between NGF stimulated and non-stimulated cells even with MK-STYX overexpression. Additionally, the localization of dopamine does not seem to differ between the experimental and control conditions. This may indicate that although MK-STYX affects the neurite extensions through internal cytoskeletal reorganization, it does not affect the levels synaptic transmission between PC12 cells. However, it may be worth examining the presence and localization of markers of synaptic vesicles like synaptotagmin and SV2 to further validate these results (Jeon, 2010).

Neurons

While model systems provide important clues, the best way to examine a protein's function in neuronal cells is to study neurons. Our overexpression studies with primary neurons revealed and validated important concepts we had seen earlier. We tried two different methods for neuronal transfection: standard Lipofectamine transfection that we use for PC12 cells and electroporation. Our transfection efficiency was higher with the electroporation method. Electroporation relies on a delivery of a high voltage shock to disrupt the plasma membrane of the cell, which creates pores to allow DNA or other transformative agent to enter the cell. Although this method has drawbacks, it is a reliable method to ensure high transfection efficiency (Potter, 2003). Neurons overexpressing MK-STYX showed distinct characteristics from the control cells. They had more significantly more neurite extensions coming from the cell body. Additionally, the cells seem to lack one long extension (presumably the axon) but had many extensions of approximately the same length. This indicates that MK-STYX may be disrupting some mechanism for axonal formation or LKB1 function. Additionally, we noticed that MK-STYX was localizing to the cell body as well as the extensions whereas, our control vector was present mostly in the cell body. This verifies our results with PC12 cells about MK-STYX affecting cytoskeleton-related proteins along neurite extensions.

There were a few interesting things that we noticed in the cell culture with MK-STYX overexpression. First, the extensions coming from a cell seemed to create a web of connections with extensions from other cells. In normal neuronal cells, extensions from the same cell do not overlap due to hemophilic binding and other avoidance mechanisms. However, for cells expressing MK-STYX, the extensions were crisscrossing and growing on top of one another. Could MK-STYX also be affecting self-recognition proteins along the extensions? Second, cultures overexpressing MK-STYX showed a higher number of cells per area but also had more

debris in the culture than the control cells. The survival of these cells is regulated by apoptotic factors. Previous research has shown that RNAi mediated knockdown of MK-STYX prevented mitochondrion-dependent apoptosis (intrinsic apoptosis). MK-STYX regulates apoptosis in a positive manner (Nieme, 2011). Our results show a lot more debris in our cell culture samples, which could indicate that overexpression of MK-STYX is perhaps inducing more cells to die. Although we don't have a clear understanding of the mechanisms behind this MK-STYX association with apoptosis, it is apparent that based on previous research and our current research that MK-STYX does cause changes in cellular survival across different cell lines.

Future Directions

Our studies mainly focus on overexpression of MK-STYX and observing the outcome in the hopes of finding pathways or interacting partners. By the same token, we also need to take the time to study the effects of a MK-STYX knockdown and seeing which pathways are disrupted or what cellular function goes awry.

Future Aim 1: Previous research has shown that MK-STYX affects RhoA activity, thereby affecting the RhoA pathway. Does MK-STYX interact directly with or work upstream of RhoA? To answer this, we can perform co-immunoprecipitation assays with MK-STYX and RhoA and see if there are any interactions. We can also immunostain for RhoA and compare its localization relative to MK-STYX or the control.

Cofilin is a downstream effector of RhoA and its phosphorylation state is partially dependent on RhoA activity. Cofilin's phosphorylation state affects cytoskeletal dynamics by way of actin filament stability. When cofilin is phosphorylated, actin filaments are stabilized and there is no net movement forward. When cofilin is dephosphorylated, actin monomers are removed from the filament and the filament destabilizes. The destability lends itself to a net

movement of the filament in one direction. However, phosphorylated and unphosphorylated cofilin cycle actin filament stability and instability (Huang, 2006). Therefore, it may be worthwhile to examine how MK-STYX temporally affects the phosphorylation states of cofilin.

It is also important to question whether MK-STYX is regulating cofilin phosphorylation via other enzymes. Is MK-STYX binding directly to cofilin and preventing a change in phosphorylation state? MK-STYX could also be interacting with cofilin's kinase, LIMK, or cofilin's phosphatase, Slingshot. Studying the interaction of these proteins with MK-STYX by co-immunoprecipitation and more localization studies will be helpful. Studies further down the road can also explore the kinases, phosphatases and scaffolding enzymes associated with slingshot.

Future Aim 2: While PC12 cells have served as excellent model systems, there is no better way to study the effect of MK-STYX on neuronal systems than by actually using neurons. Our studies with neurons only scratch the surface in terms of MK-STYX's effect. They have focused primarily on morphological changes and localization of MK-STYX in the cell. Does MK-STYX also impact the number of connections between neurons, the length of the extensions, the type and amount of neurotransmitter being exchanged? The next studies will focus on answering these questions and understanding how different signaling cascades are impacted.

Future Aim 3: We are also undertaking projects in collaboration with Dr. Anne Claude-Gingras at the Lunenfeld-Tanenbaum Research Institute of Mount Sinai Hospital. Preliminary studies with overexpression of MK-STYX in N2A (neuro2A cells derived from mouse neuroblastoma) cells under retinoic acid stimulation showed more extensions coming from the cell body (Hinton et al., unpublished data). These results indicate that MK-STYX has an effect on neuronal cell not only across different cell lines but also across various species. Exploring overexpression and

knockdown, protein interactions, and more quantitative data on morphological changes will serve well to understanding MK-STYX. Additionally, recent affinity purification mass spectrometry data shows that MK-STYX with six cytoskeletal proteins. Understanding these interactions in neuronal cells will be instrumental for possible therapies for neurodegenerative disorders.

EXPERIMENT FIGURES

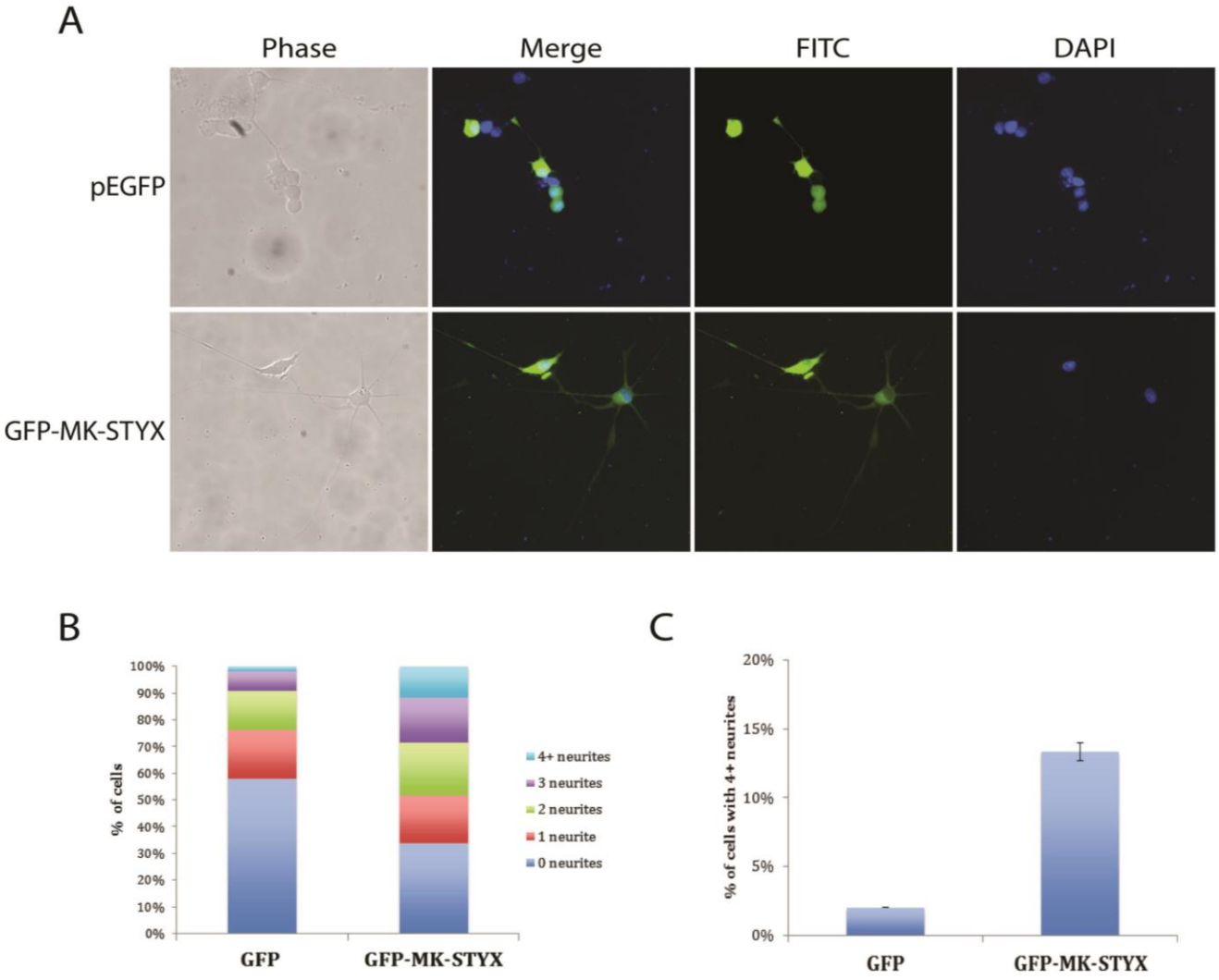

Figure 6. MK-STYX induces neurite outgrowths in the presence of NGF

(A) Live PC-12 cells overexpressing GFP-MK-STYX or pEGFP control plasmid for 24 h were stimulated with 100 ng/ml NGF. Seventy-two hour post-stimulation cells were imaged for primary neurites (extensions from cell body) ≥20 μm, and representative images were taken. (B) The number of primary neurites ≥20 μm in PC-12 cells overexpressing pEGFP or GFP-MK-STYX plasmids in the presence of NGF was scored (n = 50). Statistical analysis was performed

(unpaired t-test: $p < 0.05$, 3; $p < 0.0005$, 4+ neurites). (C) Cells overexpressing GFP-MK-STYX significantly $p < 0.0005$ increased primary neurites by four or more.

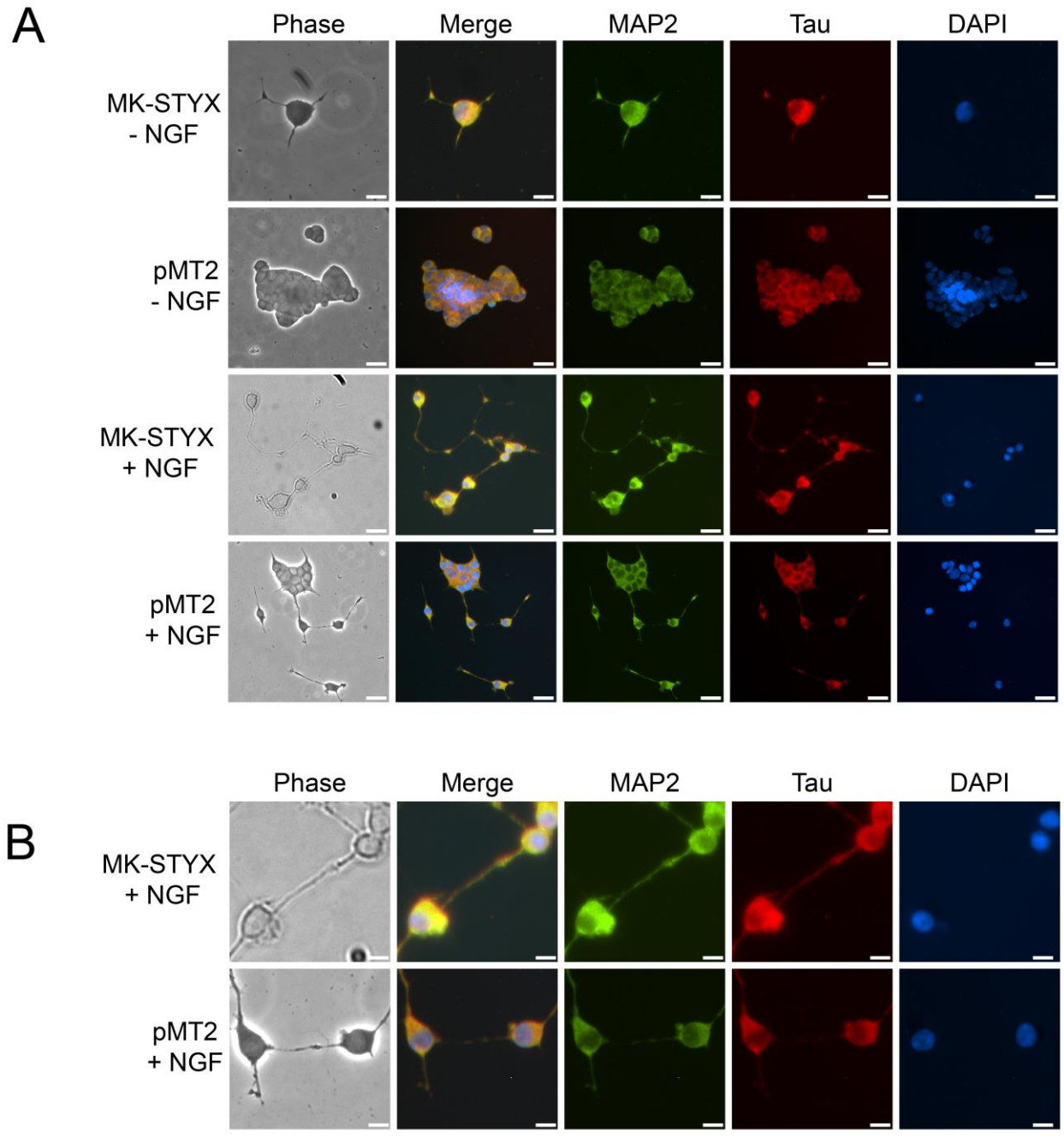

Figure 7. MK-STYX affects MAP2 and Tau localization

(A) PC-12 cells transfected with MK-STYX or control pMT2 plasmid vector were incubated in the presence or absence of NGF for seventy-two hours and stained with the dendritic MAP2 marker and the axonal Tau marker. Representative examples of MAP-2 immunoreactivity (green) show it in the cell body in non-stimulated cells, but also in major processes in stimulated cells or cells over-expressing MK-STYX. Tau (red) was localized to the cell body in non-stimulated cells, whereas it was more intense in neurites in NGF stimulated cells or MK-STYX expressing cells, demonstrating that stimulated control cells and MK-STYX expressing cells have pre-synaptic (axons-Tau) and post-synaptic (dendrites-MAP2) processes. **(B)** In a higher magnification view of stimulated cells, the connections between the presynaptic and post-synaptic cells are visible and possibly enhanced in cells expressing MK-STYX. Cells were fixed, stained with anti-MAP2 and Cy5-conjugated goat anti-rabbit antibodies, anti-Tau and Cy3-conjugated goat anti-mouse antibodies, and DAPI, and analyzed 72 hr post-stimulation by fluorescence microscopy. Merged images show the localization of MAP2 (green), Tau (red) and DAPI-stained nuclei (blue). Five replicate experiments were performed.

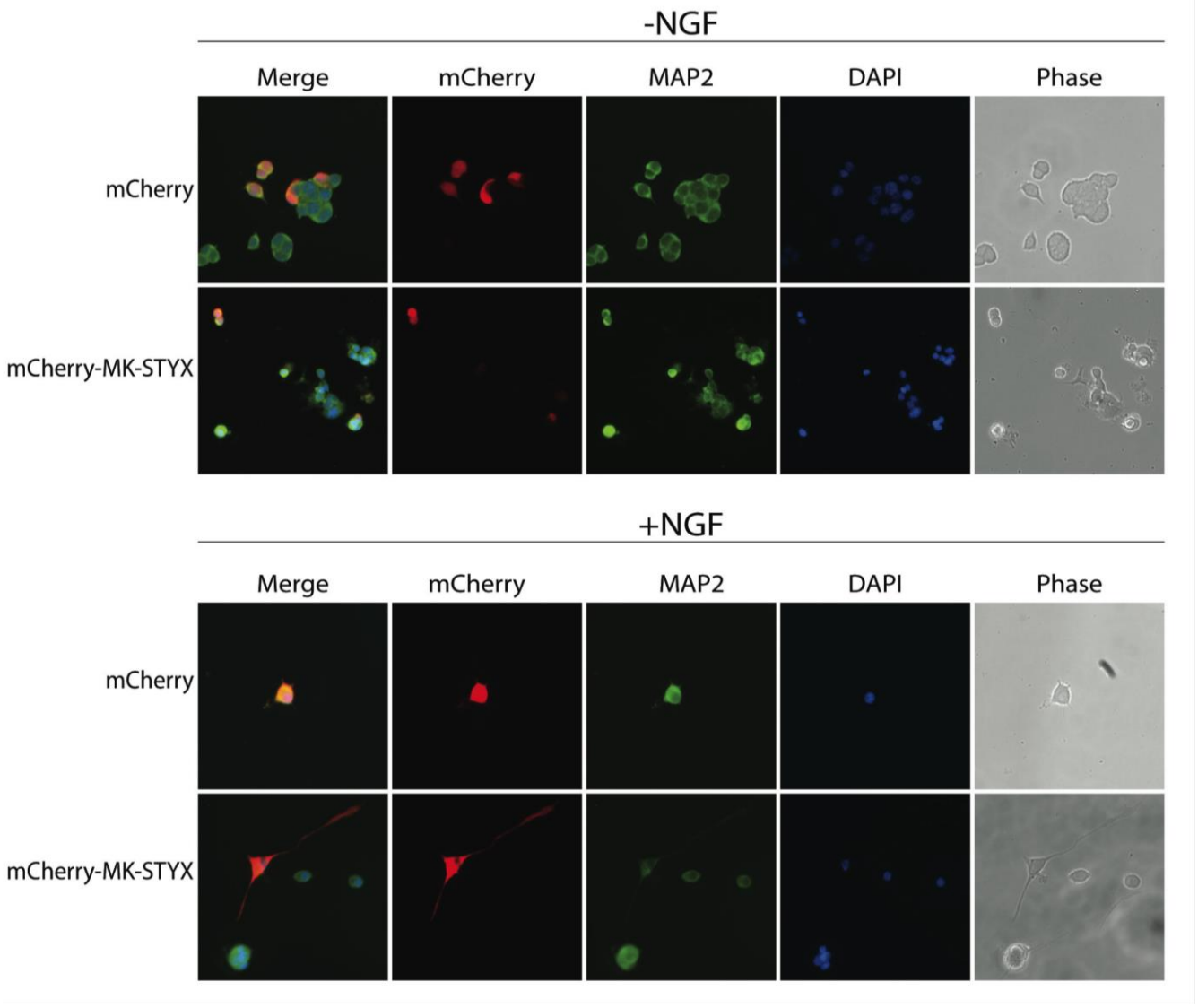

Figure 8. MK-STYX co-localizes with MAP2

PC12 cells were imaged using phase and fluorescence microscopy after transfection with mCherry or mCherry-MK-STYX to illustrate the localization of dendritic marker MAP2. The top panel shows cells without NGF stimulation whereas the bottom panel shows cells with NGF stimulation. In cells transfected with mCherry-MK-STYX and stimulated with NGF, MAP2 and MK-STYX colocalize with each other, especially in the neurite extensions. Three replicates of this experiment were performed.

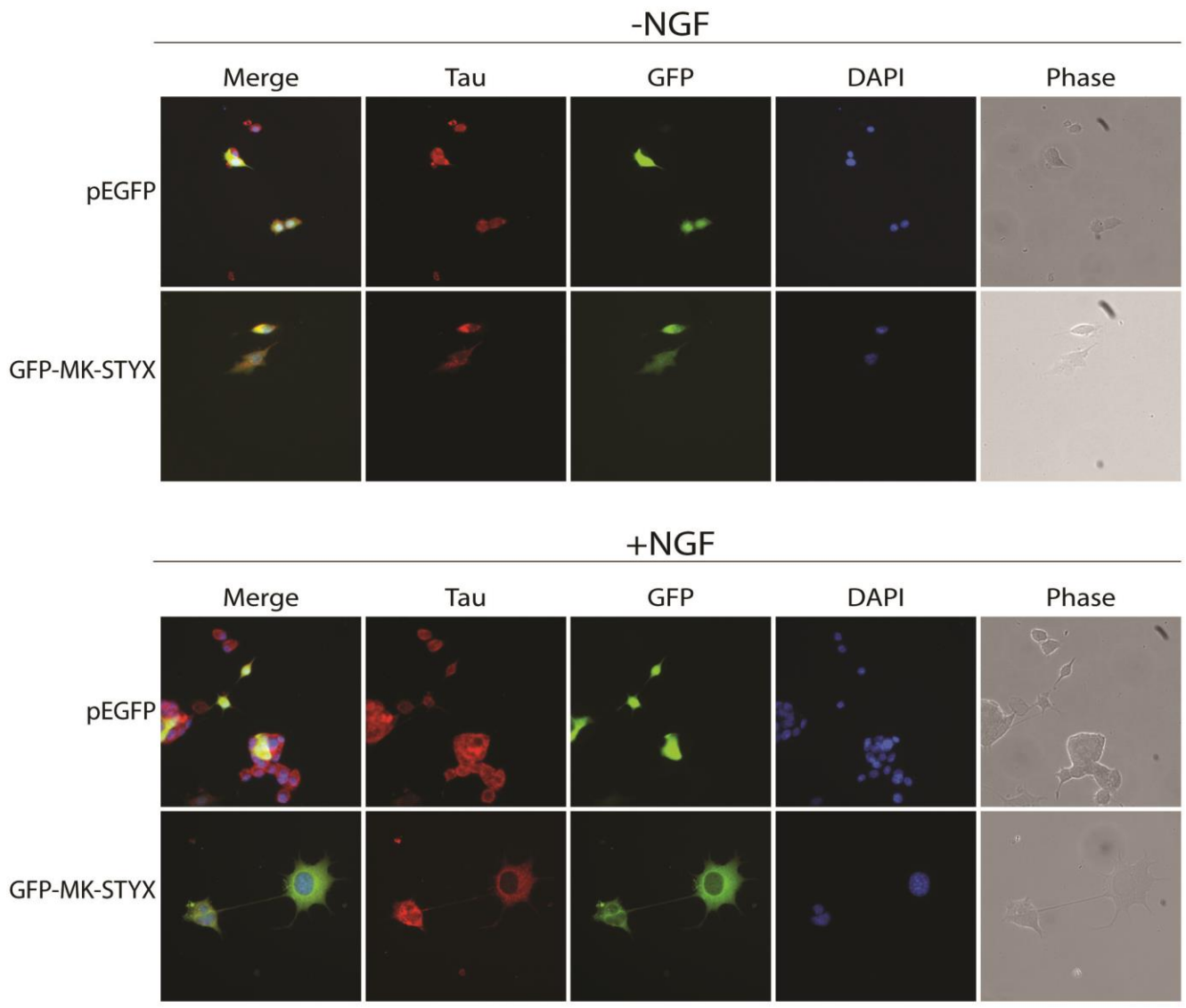

Figure 9. MK-STYX localizes to the same cellular compartment as Tau

PC12 cells were imaged using phase and fluorescence microscopy after transfection with GFP or GFP-MK-STYX to illustrate the localization of axonal marker Tau. The top panel shows cells without NGF stimulation whereas the bottom panel shows cells with NGF stimulation. In cells transfected with GFP-MK-STYX and stimulated with NGF, Tau and MK-STYX seem to colocalize with each other, especially in the neurite extensions. Three replicates of this experiment were performed.

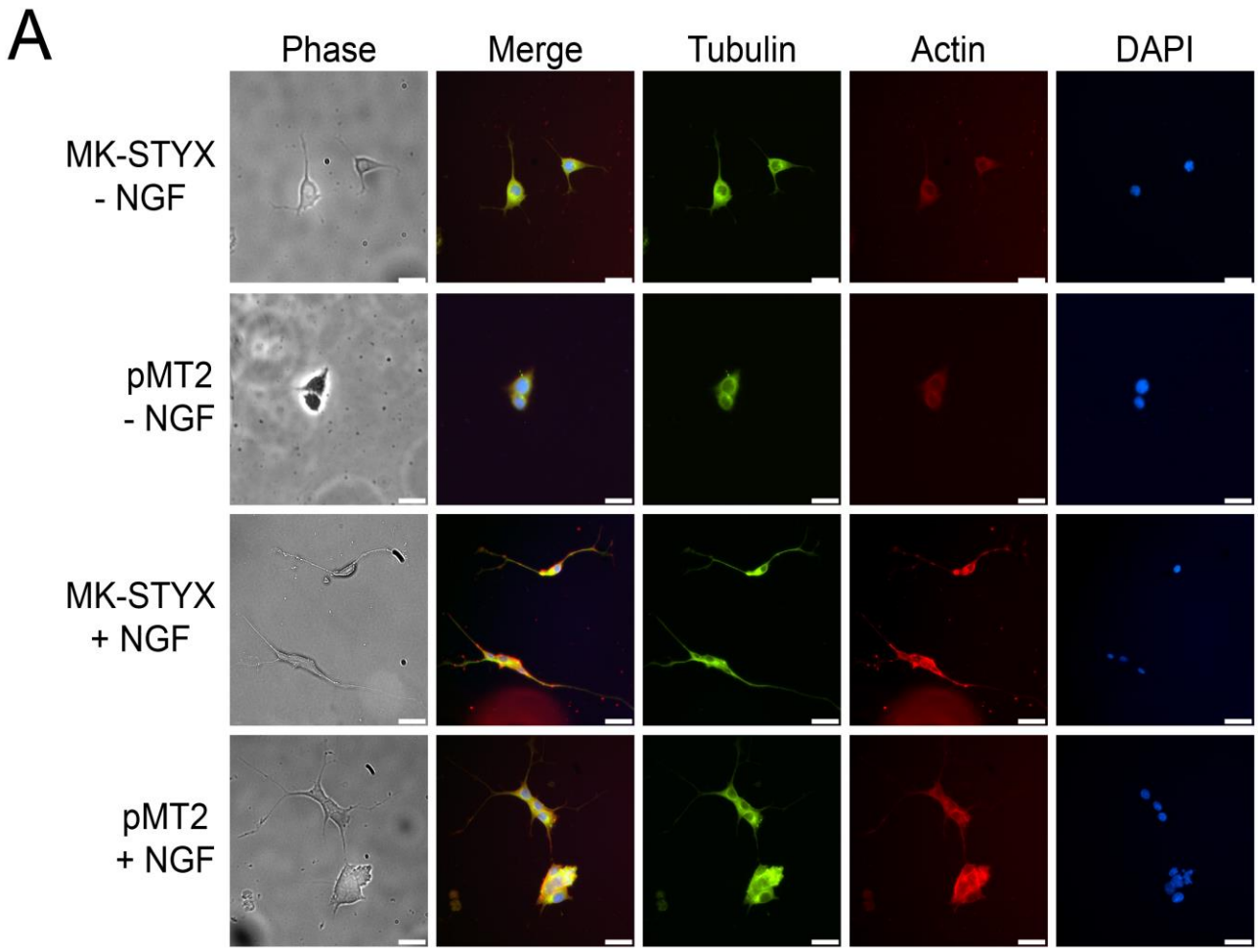

Figure 10. MK-STYX affects actin and tubulin in PC12 cells

PC12 cells were imaged using phase and fluorescence microscopy after transfection with pMT2 or GFP-MK-STYX. Post 72 hours NGF stimulation, cells were fixed and sequentially stained with rhodamine-conjugated phalloidin (actin), anti-β-tubulin conjugated FITC antibody, and DAPI. Cells with MK-STYX overexpressed show bundles of actin at the end of the neurite extensions. Three replicates of this experiment were performed.

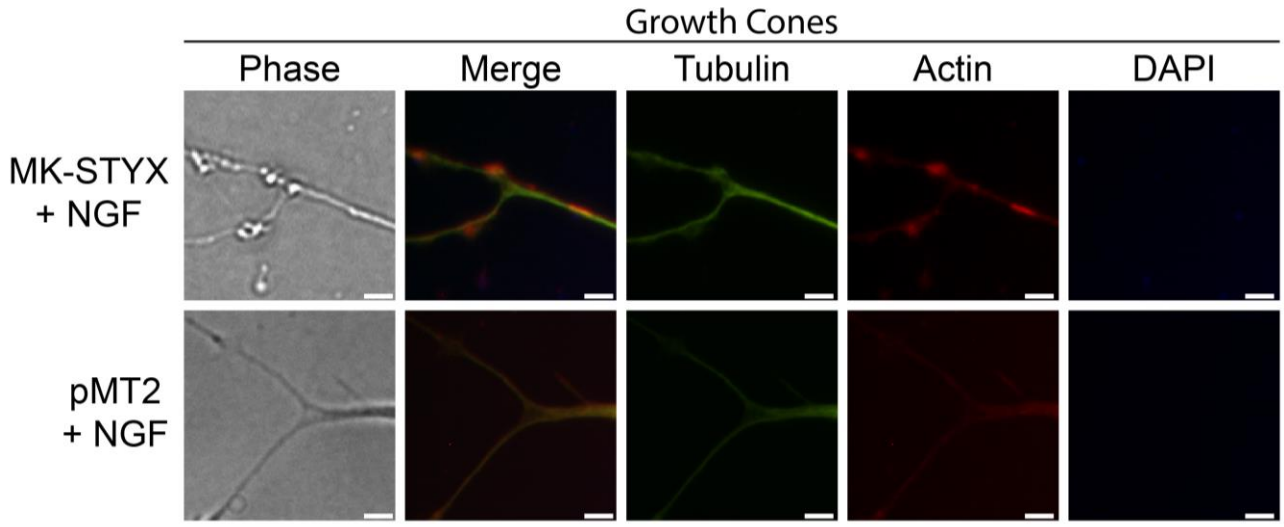

Figure 11. MK-STYX induces actin-rich protrusions in PC12 cells

PC12 cells were imaged using phase and fluorescence microscopy after transfection with pMT2 or GFP-MK-STYX. Post 72 hours NGF stimulation, cells were fixed and sequentially stained with rhodamine-conjugated phalloidin (actin), anti-β-tubulin conjugated FITC antibody, and DAPI. Three replicates of this experiment were performed. Actin-rich puncta along the length and the ends of the microtubule-rich neurites indicate active growth cones.

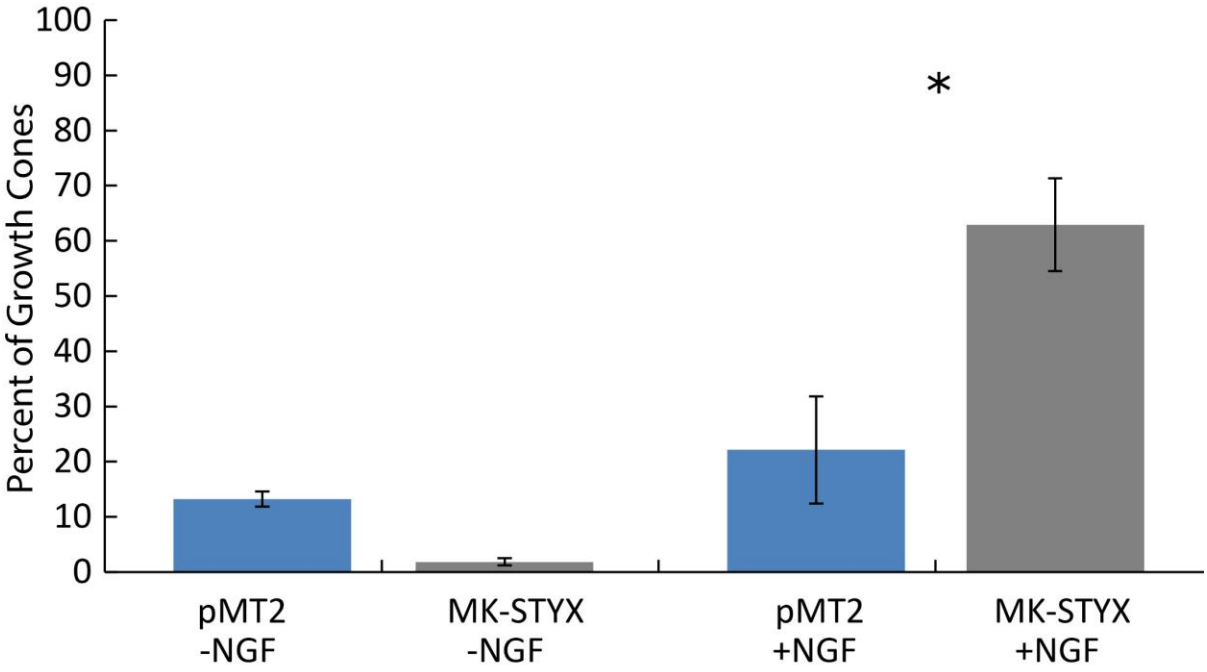

Figure 12. MK-STYX significantly increases growth cones for cells under NGF stimulation

Live PC12 cells were transfected with pMT2 or GFP-MK-STYX. Post 72 hours NGF stimulation, cells were fixed and sequentially stained with rhodamine-conjugated phalloidin (actin), anti-β-tubulin conjugated FITC antibody, and DAPI. Three replicates of the study were performed. The slides were initially blinded so no researcher bias could affect the results. Out of one hundred cells, the number of cells that had growth cones was manually counted. The cells chosen were located all over the slide. Statistical analysis was performed (unpaired t-test: $p < 0.05$, pMT2 with NGF and MK-STYX with NGF). Error bars represent $\pm$ SEM.

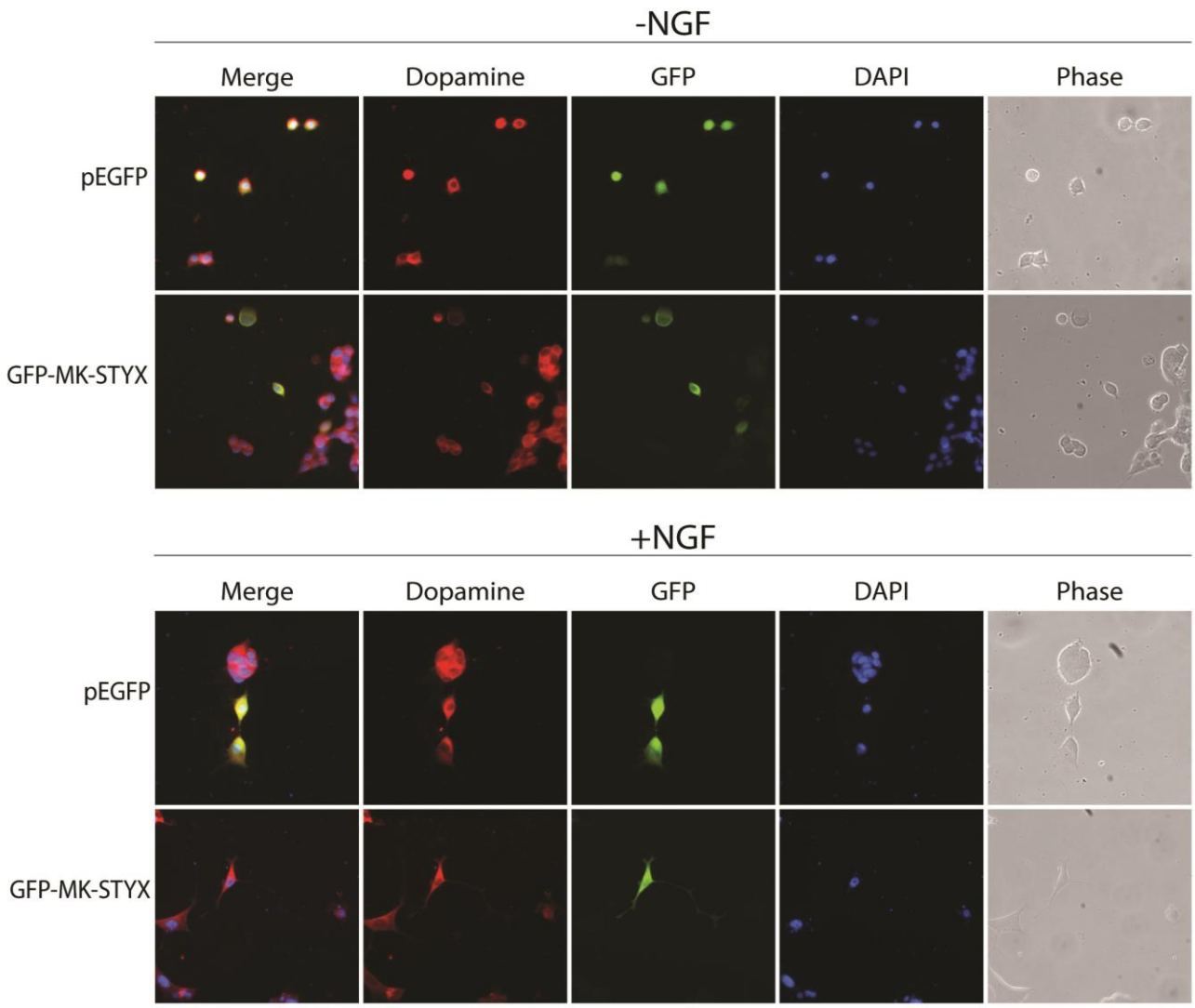

Figure 13. MK-STYX does not affect dopamine localization

PC12 cells were imaged using phase and fluorescence microscopy after transfection with GFP or GFP-MK-STYX to illustrate the localization of the neurotransmitter dopamine. The top panel shows cells without NGF stimulation whereas the bottom panel shows cells with NGF stimulation. Dopamine localization was not significantly affected by MK-STYX overexpression.

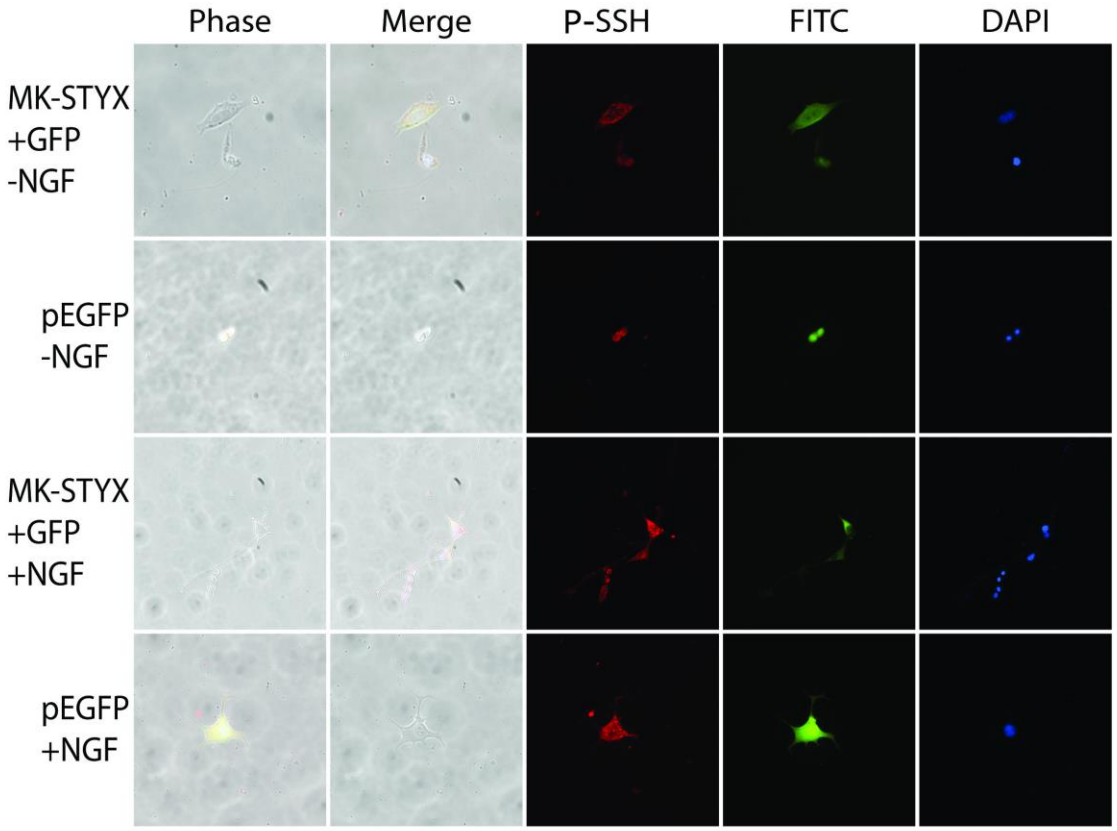

Figure 14. MK-STYX does not affect phospho-slingshot localization

PC12 cells were imaged using phase and fluorescence microscopy after transfection with GFP or GFP-MK-STYX to illustrate the localization of phosphor-slingshot. The top panel shows cells without NGF stimulation whereas the bottom panel shows cells with NGF stimulation. Phospho-slingshot localization was not significantly affected by MK-STYX overexpression.

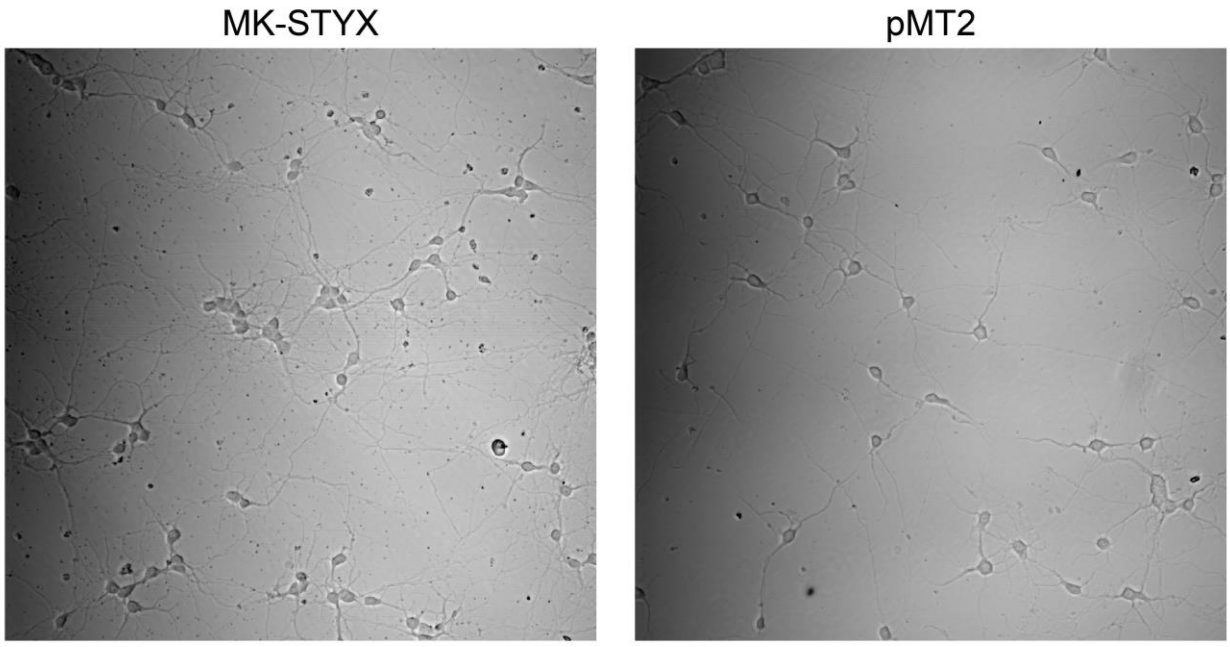

Figure 15. MK-STYX changes neuronal survival patterns
Live neurons were imaged using phase microscopy to illustrate the growth of neurite extensions in cells three days after transfection with pMT2 or MK-STYX. Cells transfected with MK-STYX seemed to have more extensions coming from the soma compared to the control. Cultures with cells transfected with MK-STYX showed more cells per area and also showed more debris. Three replicate experiments were performed.

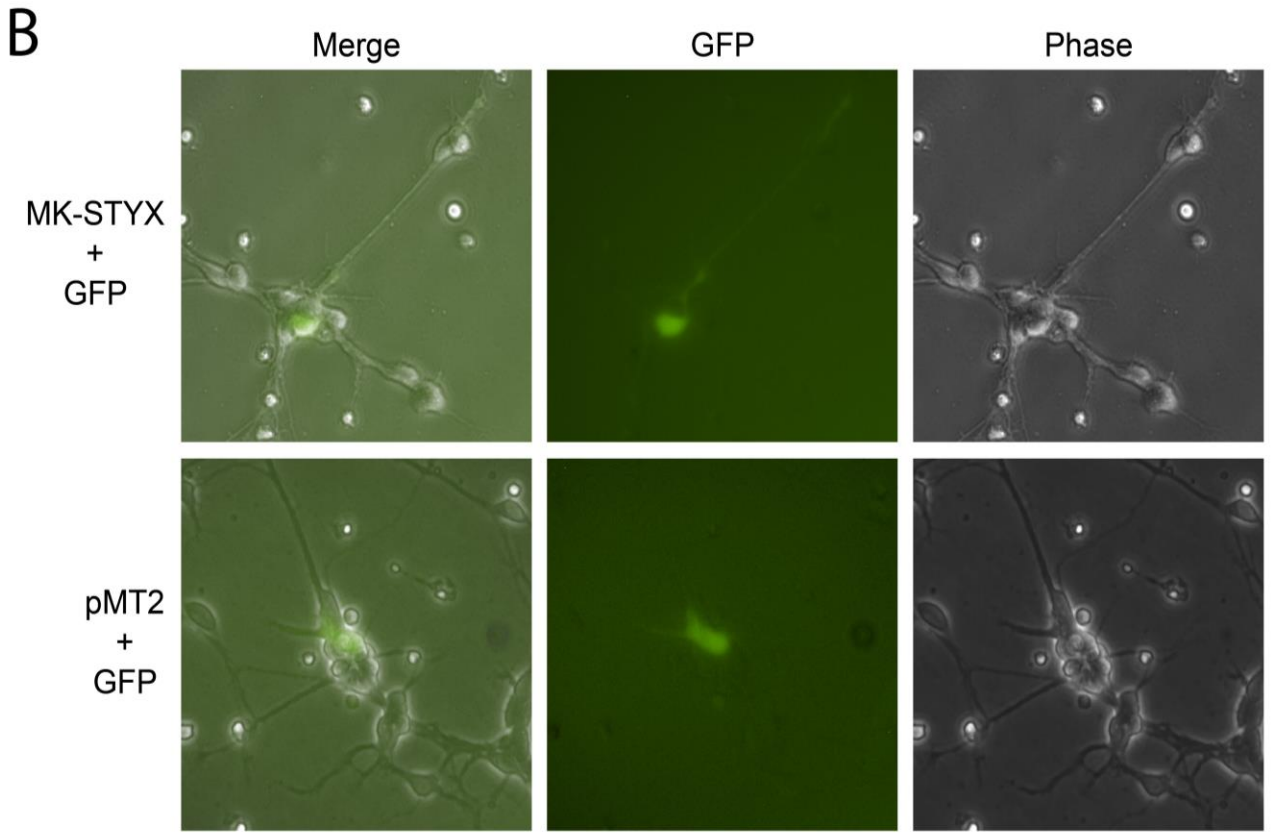

Figure 16. MK-STYX localizes in neuronal extensions

Live neurons were imaged using phase and fluorescence microscopy to illustrate the growth of neurite extensions in cells co-transfected with GFP and either pMT2 or MK-STYX. GFP was localized in the neuronal extensions in the cells transfected with MK-STYX whereas the GFP localized in the cell body when co-transfected with pMT2. Three replicate experiments were performed.

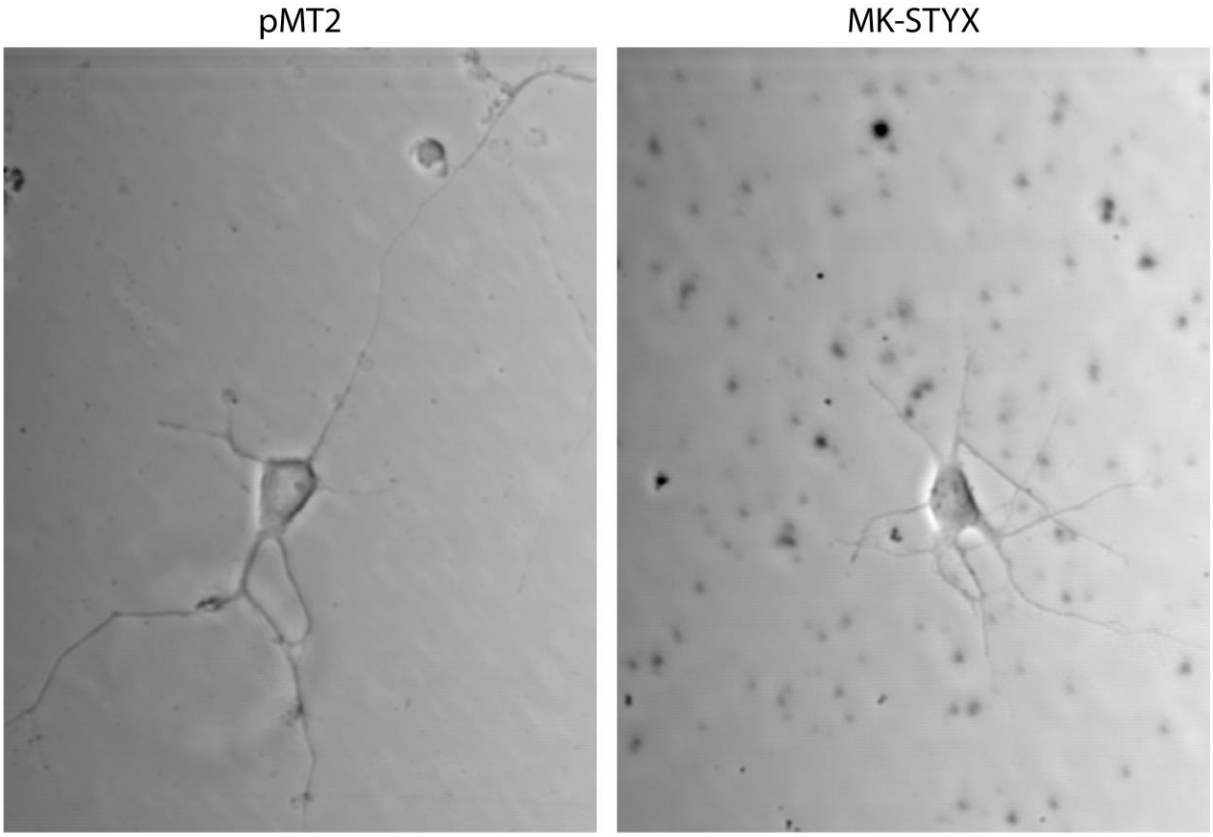

Figure 17. MK-STYX induces more outgrowths in neurons

Live neurons were imaged using phase microscopy to illustrate the growth of neurite extensions in cells transfected with pMT2 or MK-STYX. Cells transfected with MK-STYX had more extensions coming from the soma compared to the control. Three replicate experiments were performed. Cultures with cells transfected with MK-STYX also showed more debris. Three replicates of this experiment were performed.

APPENDIX

Immunostaining buffer preparation

PC12 cell lines do not adhere well to each other or their surface and they require extra measures for adhesion. Along with collagen cover slips, phosphate buffered saline solution (D-PBS) with calcium and magnesium ions can also promote adhesion. They are divalent cations that are essential for protein (such as integrin) function. Therefore, Ca_2 and Mg_2 are important for cell-to-cell contact and improve connections within the extracellular matrix. They also improve chromatin visualization and reduce DAPI bleed-though.

The following solutions should be made and autoclaved separately.

Preparation of 10X D-PBS: Bring to 1 Liter with ddH2O

- 2 grams KCl for a final concentration of 27 mM
- 2 grams $KH_2PO_4$for a final concentration of 14.7 mM
- 80 grams NaCl for a final concentration of 1370 mM
- 21.6 grams $Na_2HPO_4 \circ 7H2O$ for a final concentration of 162 mM

Preparation of 10X $CaCl_2$: Bring to 100 mL with ddH_2O

- 1 gram $CaCl_2$ for a final concentration of 9.01 mM

Preparation of 10X $MgCl_2$: Bring to 100 mL with ddH_2O

- 1 gram $MgCl_2 \circ 6H2O$ for a final concentration of 4.92 mM

Preparation of 1X D-PBS with 100mg/L $CaCl_2$ and $MgCl_2$:

- Take 100 ml of 10X PBS, bring to 800 mL with dI water
- Take 10 ml of 10X $CaCl_2$, bring to 100 mL with dI water
- Take 10 ml of 10X $MgCl_2$, bring to 100 mL with dI water

Take 800 ml of DPBS and slowly add $MgCl_2$ and $CaCl_2$, swirling the solution to dissolve any precipitate. Don't add the $CaCl_2$ first because it reacts with the phosphate in the D-PBS and forms a precipitate.

PC12 immunostaining with formaldehyde

PC12 cells tend to wash away easily when immunostaining, even if they are affixed to collagen-covered cover slips. Extra care must be taken to make sure they adhere to the slips during the process. When handling PC12 cells for staining, always use serological pipettes attached to 1000 µl pipet tips attached to 200 µl pipet tips. Remember to also pipet up and down the sides of the well as opposed to directly on top of the coverslip. This will reduce the force of the liquid on the cells. PC12 cells are immunostained under standard immunostaining protocol with formaldehyde, 0.2% Triton, and the antibodies. However, they are also prefixed with 3.7% formaldehyde as that increases their adherence.

Prefixation

Prepare a 3.7% formaldehyde solution in 1X DPBS with $CaCl_2$ and $MgCl_2$ with at least 3.5 ml per well. Once you take out the cells from the incubator, don't remove the media for the prefixation. Add 1.5 ml of the prepared formaldehyde solution to each well and let sit for 5

minutes. Remove media + formaldehyde from the wells and go on to fixation, permeabilization and probing.

Neurons plating with poly-D-lysine and laminin

Laminin sticks to PDL whereas neurons stick to laminin.

Materials

- PDL aliquots (in TC fridge)
- Laminin aliquots (in TC fridge)
- PDL storage -20
- Laminin storage (according to instructions)
- 2-15 ml conical tube
- 96 well plate
- Water

Poly-D-Lysine

1. Wipe down hood, follow standard TC procedure
2. In a 15 ml conical tube, put 7.6 ml of water and 75.6 μl of PDL from aliquot
3. Put 75.6 μl of mix in each well
4. Store in fridge overnight

Laminin

1. Next day, pour out poly-D-lysine, rinse 3X with 100ul of water per well
2. In a 15 ml conical tube, put 6 ml of H2O and 53 μl of laminin from aliquot
3. Put 60 μl of laminin in each well
4. Store in incubator for at least 4 hours. Prior to use, make sure the bottom is dry.

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